Identification of Early Biomarkers of Neoplastic Transformation in Mouse Tumour Models of Breast Carcinogenesis

Thesis

Identification of early biomarkers of neoplastic transformation in mouse tumour models of breast carcinogenesis

A thesis by

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Abstract

Tumour development is not an entirely cell-autonomous process, being dependent on the ability of bystander cells, mostly of bone marrow (BM) origin, to establish a pro-tumourigenic microenvironment. We hypothesize that identification of early BM changes would confirm that BM senses the onset of a neoplastic lesion in peripheral tissues even at very early stages of carcinogenesis and that such peripheral alterations could offer the opportunity to identify key signalling molecules that may represent early biomarkers of cancerogenesis and could allow discovering novel potential therapeutic targets.

To test such hypothesis we took advantage of the MMTV-NeuT (NeuT) transgenic mouse model of mammary carcinogenesis and found that profound BM modifications in the composition and spatial arrangement of the hematopoietic populations, such as increase of immature myeloid granulocytic cells, contraction of B lymphoid pools, displacement of BM niches characterize NeuT mice with overt carcinomas in comparison to wild-type mice and that such modifications were already detectable at earlier stages of carcinogenesis, although less significant.

Genes differentially expressed in the BM between transgenic and controls with overt tumour have been used to design an ad hoc "late gene signature" that has been tested and validated in two additional breast cancer models. Early NeuT BM samples already show differentially expressed genes in comparison to controls and they are efficiently separated by the late gene signature in two clusters: those with normal/mild dysplasia in their mammary glands from those with sever

dysplasia/early carcinomas. Different circulating microRNA profiles characterized early stages of tumourigenesis and BM down-regulated genes are among their predictive targets.

With an unprecedented level of integration between BM mRNA and microRNA profiles, BM immunolocalization, circulating microRNA profile and primary lesion histopathology we could demonstrate that the BM is an early sensor of incipient mammary tumour and that the alterations observed in advanced carcinomas are, at least in part, already present at early stages.

Acknowledgments

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Abbreviations

ATF3: activating transcription factor 3

B6: C57BL/6

BC: breast cancer

BM: bone marrow

CAF: cancer-associated fibroblast

CMP: common myeloid progenitors

ECM: extracellular matrix

EDTA: ethylene-diamine-tetraacetic acid

ER: estrogen receptor

ES: enrichment score

EtOH: ethanol

FC: flow cytometry

FCS: fetal calf serum

FDR: false discovery rate

GEP: gene expression profile

GMP. granulocyte-monocyte progenitors

GO: gene ontology

GSEA: gene set enrichment profile

H&E: haematoxylin & eosin

IHC: immunohistochemistry

MEP: megakaryocyte-erythroid progenitors

MDSC: myeloid-derived suppressor cells

MMP: matrix metalloproteinase

NeuT: Tg(MMTV-Erbb2)1Pv

PCR: polymerase chain reaction

PBL: peripheral blood

PBS: phosphate-buffered saline

PyMT: C57BL/6-Tg(MMTV-PyVT)634Mul/J

PR: progesterone receptor

RIN: RNA integrity number

RNA: ribonucleic acid

SD: standard deviation

SEM: standard error of the mean

SPL: spleen

TDLU: terminal ductal-lobular unit

TME: tumour microenvironment

TG: transgenic

WT: wild type

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1 Introduction

1.1 Breast Cancer biology

1.1.1 Mammary gland organization

The adult female breast is composed of epithelial lactiferous ducts terminating in secretory alveoli embedded in a complex microenvironment comprised of basement membrane, extracellular matrix (ECM) molecules and a variety of stromal cell types including adipocytes, fibroblasts, endothelial cells and immune cells. The functional unit of the mammary gland is represented by the lobus, which is drained by a duct and it is divided into smaller ducts ending with the terminal ductal-lobular unit (TDLU). Each TDLU is composed of two kinds of cells organized in distinct layers: luminal epithelial cells, expressing CK8, surrounded by CK5+ basal myoepithelial cells, lying on the basal membrane (Figure 1).

In response to estrogen, progesterone and other hormones, the developing ducts begin to infiltrate into the surrounding stromal tissue, a mechanism that requires extensive remodeling of the ECM. This process of ECM remodeling, together with proliferation and migration of mammary epithelial cells, is termed branching morphogenesis (Lu and Werb 2008). Normal breast growth and development are regulated by a complex interaction of many hormones and growth factors such as prolactin, thyroid hormone, insulin, insulin-like growth factors (IGF) 1-2, fibroblast growth factor (FGF), endothelial growth factor (EGF), transforming growth factor alpha (TGF-α) (Sternlicht, Kouros-Mehr et al. 2006).

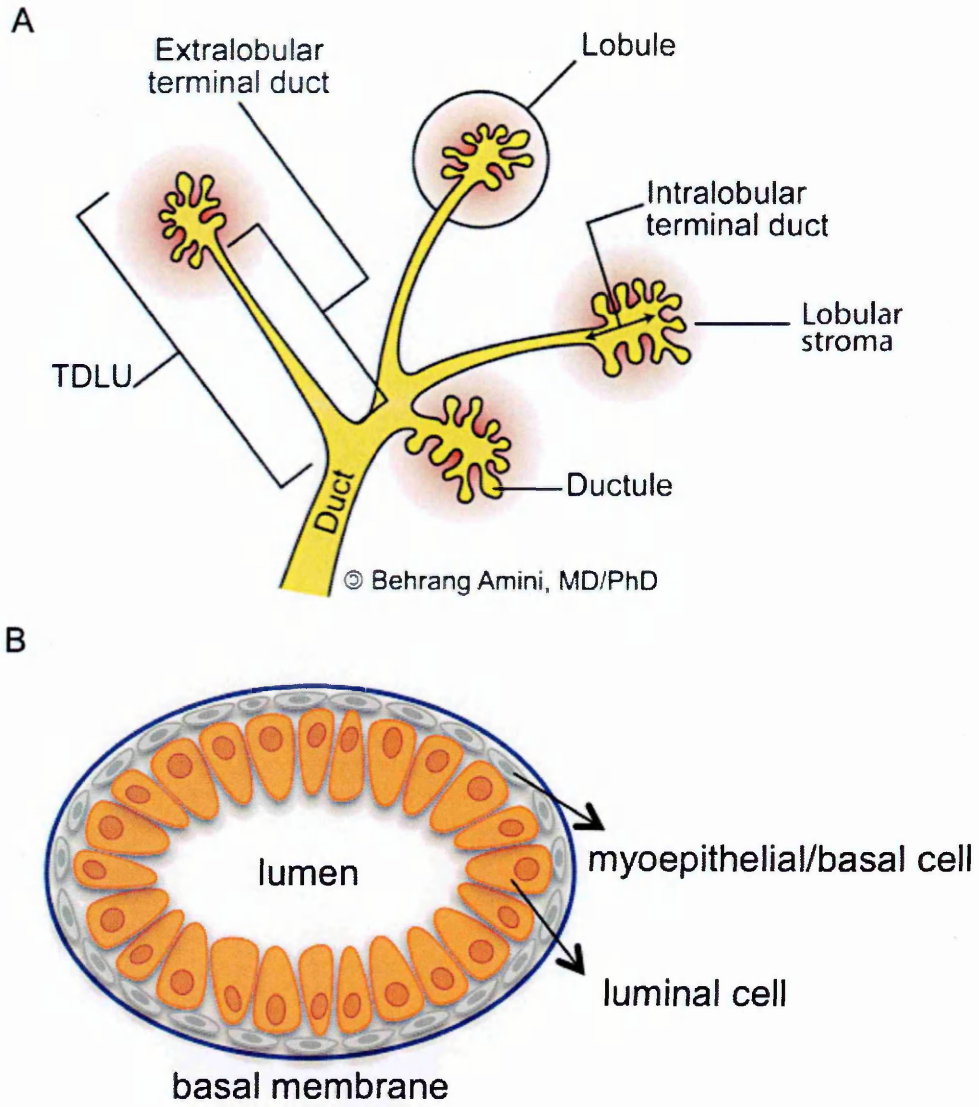

Figure 1 - Schematic representation of the mammary gland structure.

A) Pre- and pubertal mammary glands are composed by duct terminating in terminal end buds (TEBs); B) in a normal mammary gland alveoli are formed by a single layer of luminal cells surrounded by myoepithelial cell and a basement membrane, providing contact with the extracellular environment.

All these molecules can act in an autocrine manner if secreted by mammary cells themselves or in a paracrine way when secreted by stromal accessory cells. Such cross-talk between the stromal microenvironment and mammary epithelial cells actively contributes to direct the mammary gland branching morphogenesis (Dickson and Lippman 1995) (Polyak and Kalluri 2010).

Among the most abundant cells present in the mammary gland stroma, adipocytes provide not only a scaffold for the branching epithelia, being indispensable for development and function of the gland, but also represent a reservoir of metabolites and signaling molecules (Hovey and Aimo 2010).

In addition to adipocytes, cells of the immune system have pleiotropic roles in mammary gland development. For instance, macrophages promote epithelial cell proliferation and chemotaxis, secreting EGF, and are necessary for ductal outgrowth (Gouon-Evans, Rothenberg et al. 2000). Mast cells coordinate epithelial proliferation and morphogenesis by releasing serine proteases by degranulation (Lilla and Werb 2010). Fibroblasts maintain the extracellular environment through the production and remodeling of the ECM (Maller, Martinson et al. 2010) (Arendt, Rudnick et al. 2010). The collagen-rich ECM provides the architecture supporting gland development and represents a physical barrier that must be remodeled to allow the branching of the ducts.

In summary, the cross-talk between multiple cell types within the epithelial and stromal compartment of the mammary gland participate in the developmental program of the mammary architecture and functionality.

1.1.2 Breast cancer development and classification

Breast cancer (BC) is the most common cancer in women with the highest mortality of any cancer worldwide. As the majority of tumours, BC is really a heterogeneous disease, with different pathologic features, biologic behaviour and variable response to therapy. Despite these differences a recent study demonstrated that most of the malignant lesions arising in the mammary gland derive from luminal progenitors (Molyneux, Geyer et al. 2010). The conversion of a normal cell of the breast into a neoplastic cell can be due to cell intrinsic changes (such as genetic alterations) or cell extrinsic, that could derive to the exposition to exogenous environmental factors.

Breast transformation could be due to multiple types of genetic alterations usually affecting three classes of genes: (i) oncogenes, genes that lead to malignancy only if activated by mutation, enhancement of expression or amplification, (ii) tumour suppressor genes, which function has to be lost by either mutation or deletion, (iii) modifiers, genes that are involved in DNA repair processes. Mutated cells start to proliferate in abnormal way originating a condition called hyperplasia. With the accumulation of other mutations, the cells also experience phenotypic variations becoming dysplastic then affecting the architecture of the tissue itself, losing their differentiation and the contact with other cells. At this stage, called carcinoma *in situ*, proliferation is confined in the tissue of origin, without the disruption of the basal membrane integrity that would constitute extension beyond epithelial boundaries. During malignant progression additional mutations may occur, enabling the tumours to cross the basal membrane invading neighbouring

tissues and to shed cells into the blood or lymphatic system, that spread to distant organs and form metastases (Figure 2).

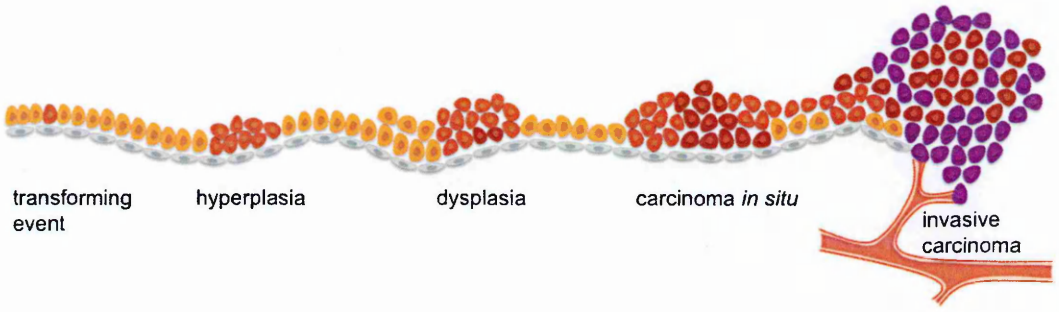

Figure 2 – Breast cancer development.

Cancer progression starts with a transforming event, usually a mutation, that makes cells more likely to divide. Transformed cells begin to grow without control creating a condition called hyperplasia. Accumulating other mutations, cells continue with their abnormal proliferation and start to change phenotype, originating a state of hyperplasia. In the next steps of tumour development, called carcinoma in situ, cells also lose their differentiation and contact, remaining in their epithelial layer of origin. The in situ cancer may remain limited or invade neighbouring tissues and shed cells into the blood or lymph, becoming a malignant invasive cancer.

Classification of BC is usually based on the histopathological appearance of the tumour tissue and on growth patterns of the tumour. In this way, BCs without invasive capacity are classified as hyperplastic lesions or *in situ* carcinoma. There are two main types of *in situ* carcinoma: ductal carcinoma in situ (DCIS) and lobular carcinoma in situ (LCIS), the latter recently considered a lobular neoplasia since it does not seem to turn into invasive cancer if left untreated (Simpson, Gale et al. 2003). Concerning invasive cancers, the most common are invasive ductal (IDC) and invasive lobular carcinomas (ILC) that together account for about 90% of invasive BC. Less common invasive tumours include inflammatory BC, a rare and aggressive form of locally advanced BC, Paget disease of the breast, a rare cancer in the skin of the nipple or closely surrounding it and metaplastic BC that is rare and difficult to diagnose. For clinical utility purpose, especially for early-stages BC, in order to have a sufficient prognostic and predictive power, a full classification should include other parameters such as grade, stage and receptor status. The grading of BC depends on the cytological similarity of BC cells to normal breast tissue and classifies the cancer as well differentiated (low-grade), moderately differentiated (intermediate grade) and poorly differentiated (high grade), reflecting progressively less normal appearance of the cells composing the tumour; high grade tumours are characterized by a worse prognosis. Moreover, in 1991, the Nottingham Grading System (NGS) has been introduced in the clinical practice becoming the recommended grading system for its prognostic relevance (Elston and Ellis 1991). In the NGS, the prognostic index is calculated combining lymph node (LN) involvement and tumour size. Finally, the TNM classification for staging BC is based on the size of the tumour (T), on tumour spreading to lymph

nodes (N) and on the presence of distant metastasis (M). In addition, along with tumour stage, the expression of hormonal receptors such as estrogen (ER) and progesterone (PR) receptors and the human epidermal growth factor receptor-2 (HER-2) are indicative to further classify BC and to drive the right therapeutic approach. These markers allow the classification in three groups: i) ER positive; ii) HER-2 overexpressing; iii) triple negative BCs (TNBC) that do not express ER, PR and HER-2. Morphologic classification, histologic grade, status of ER, PR and HER-2, along with tumour stage, is used to guide clinical management.

1.1.3 Tumour microenvironment

The tumour microenvironment (TME) is recognized as a major regulator of carcinogenesis and in BC it is well accepted that the composition of the TME could predict both prognosis and therapeutic effect (Denkert, Loibl et al. 2010)(Figure 3). As described above, breast tumours evolve via sequential progression through defined stages, starting with epithelial hyperplasia and progression to *in situ*, invasive, and metastatic carcinomas. Importantly, the cells infiltrating the tumour microenvironment have been implicated in each of these steps of cancer progression (Hanahan and Weinberg 2011). Carcinoma-associated fibroblasts (CAFs) have been demonstrated to have a role in promoting tumour progression. Accordingly, it has been shown that co-culture of normal mammary fibroblasts with BC cells can change their phenotype giving them the capability to secrete HGF, thus increasing tumour progression (Xing, Saidou et al. 2010) (Tyan, Kuo et al. 2011).

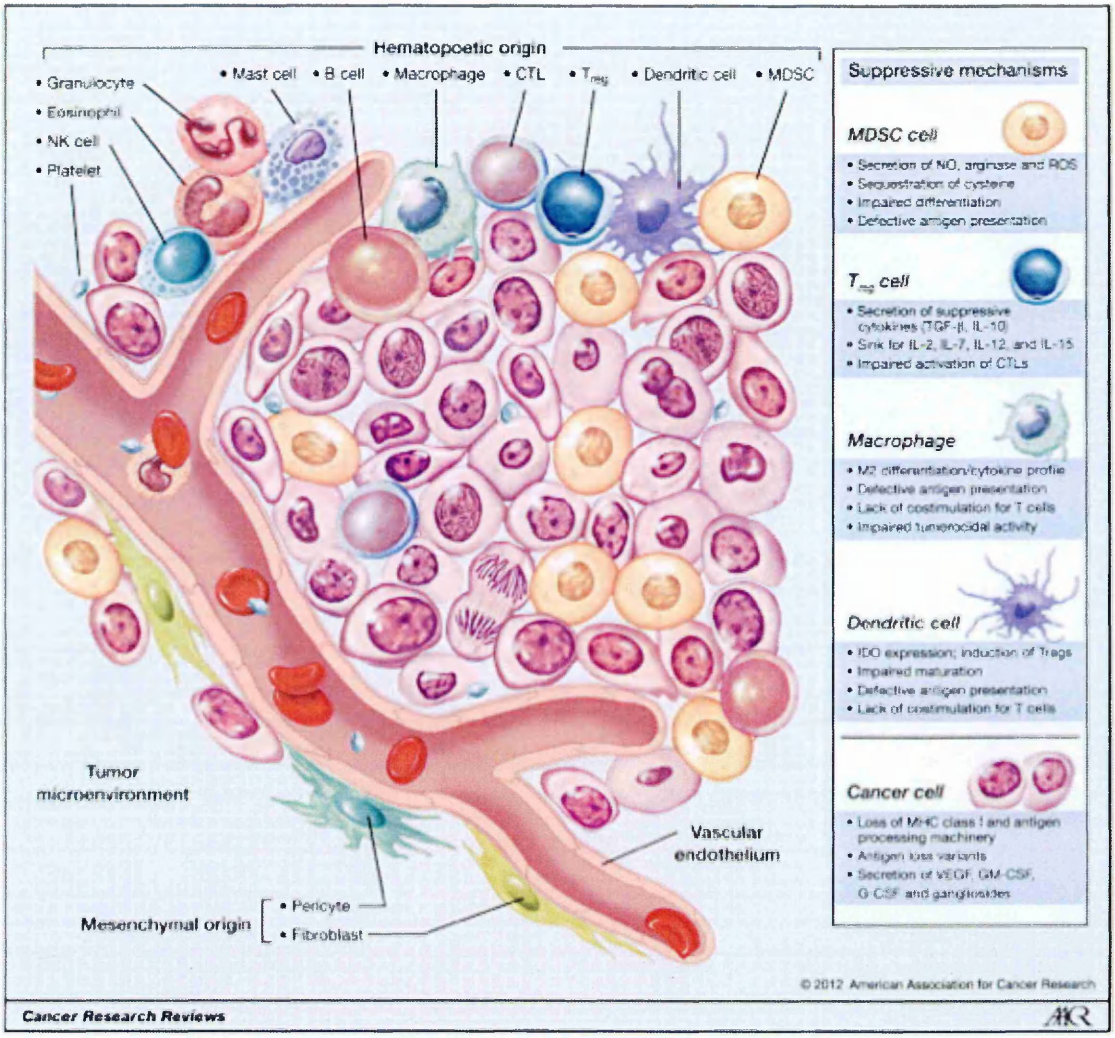

Figure 3 – The tumour microenvironment

The tumour microenvironment comprises tumour cells but also normal epithelial cells, cells of mesenchymal origin such as pericyte and fibroblasts, endothelial cells and different bone marrow-derived cells. Presence of heterogeneous cells and their secreted soluble factors, signaling molecules, extracellular matrix and mechanical cues with in the tumour microenvironment promote neoplastic transformations, support tumour growth and invasion.

From Cancer Research Reviews 2012 AACR

It seems that CAFs may originate from the differentiation of bone marrow (BM)-derived mesenchymal stem cells and be recruited to the tumour site by molecules produced by tumour cells themselves. This evidence supports the hypothesis that tumour-derived signals can induce modifications in the BM able to stimulate the generation and differentiation of cells with tumour promoting capacity (Direkze, Jeffery et al. 2006) (Shin, Nam et al. 2010). Also, BM-derived immune cells represent one of the most dynamic cell population within tumour microenvironment with both tumour promoting and inhibiting activities (Coussens and Werb 2002). For example, tumour-associated macrophages (TAM) have been shown to play a role in BC progression increasing angiogenesis, ECM degradation and consequently tumour invasion through different mechanisms (Schedin, O'Brien et al. 2007). Moreover, high numbers of infiltrating leukocytes are present in DCIS with focal myoepithelial cell layer disruptions (Man and Sang 2004), suggesting that they might play a role in invasive progression. Also, $CD4^+$ forkhead box P3 ($FOXP3^+$) T regulatory cells, $CD4^+$ T helper 2 (Th-2) cells, M2 macrophages and myeloid-derived suppressor cells (MDSCs) may promote tumour growth (Emens 2012).

Conversely, cytotoxic CD8+ T cells can exploit anti-tumour activity through different mechanism of recognition of cancer cells such as via major histocompatibility complex (MHC) class I, FAS and tumour necrosis factor-related apoptosis-inducing ligand (TRAIL) receptor. CD4+ T cells contribute to tumour eradication by the secretion of pro-inflammatory cytokines such as IL-2, IFN-γ and TNF-α that, together, promote the establishment of a type-1-like inflammation

environment that promote tumour eradication. Some of the mechanisms by which these subsets affect tumour growth are shown in Figure 4.

Moreover, tumour cells are able to suppress tumour-infiltrating lymphocytes (TILs) either through direct suppression or through the recruitment and reactivation of immunosuppressive subsets.

One of these mechanisms of suppression involves the expression of ligands for checkpoint inhibitors such as CTLA-4 and PD-1 by tumour cells that triggers the inhibitory pathways on TILs (Wolchok and Chan 2014). For example, the expression of PD-L1 on tumour cells, induces anergy/apoptosis of PD-1^+ $CD8^+$ T cells, halting the host antitumour immune responses (Pardoll 2012). Accordingly, both PD-1 and CTLA-4 blockade have been proven to be very effective, not only in preclinical animal models (van Elsas, Hurwitz et al. 1999, Fan, Quezada et al. 2014, Kim, Skora et al. 2014, Pentcheva-Hoang, Simpson et al. 2014), but also in clinical practice. Indeed, an increasing number of studies are showing positive results for such immune checkpoint blockers, at least in some tumour histotypes (Brahmer, Drake et al. 2010, Wolchok, Neyns et al. 2010, Postow, Callahan et al. 2015).

In addition to the cellular components of the tumour stroma, also the ECM of BC, markedly abnormal, has been shown to promote tumour progression. For example, matrix metalloproteinases (MMPs) are a large family of endopeptidases, which are synthesized predominantly by fibroblasts and normally participate in tissue remodelling and wound healing.

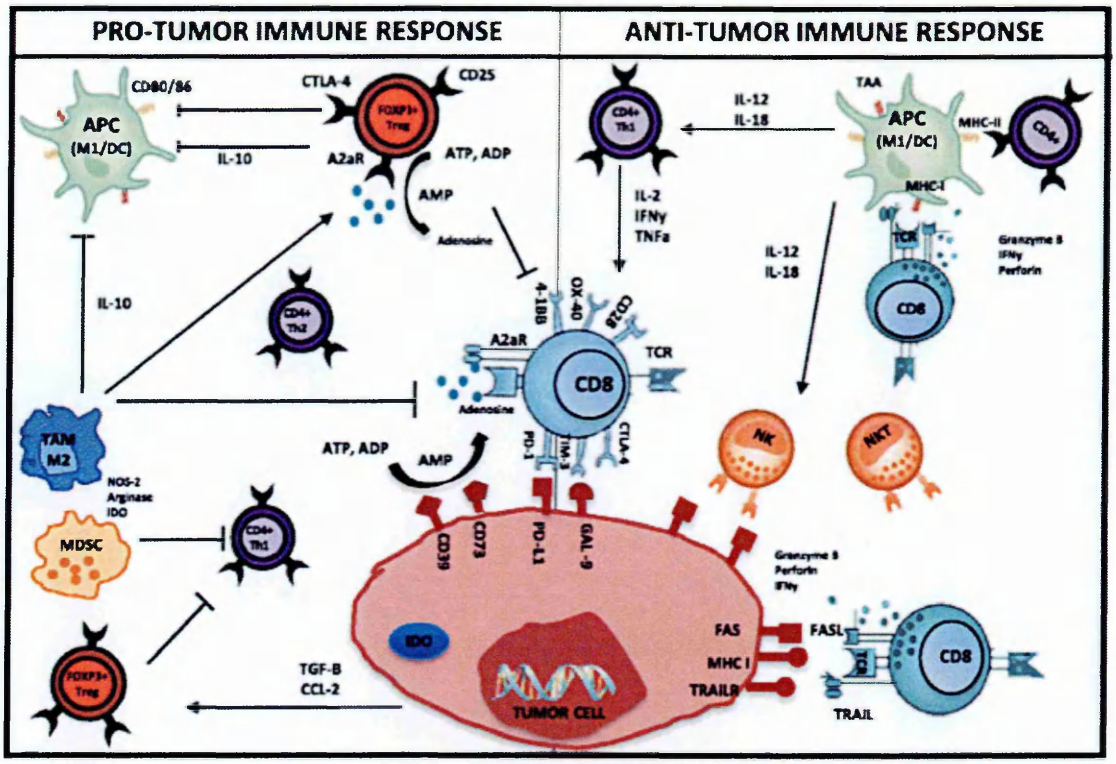

Figure 4 - Pro- and anti-tumour immune response in breast cancer.

The antitumour immune response depends on IFNγ produced by $CD4^+$ T cells responsible of the expansion, differentiation, and activation of tumour-specific $CD8^+$ cytotoxic T cells. $CD8^+$ T cells are able to recognize tumour associated antigens thus inducing lysis of the cells. Similarly, $CD4^+$ T cells recognize MHC II on antigen presenting cells. The reults of these interactions is the release of granzymes, IFNγ, and perforin from these cytotoxic lymphocytes resulting in granule exocytosis and tumour cell death via apoptosis. Also, NK and NKT cells are able to recognize and eliminate tumour cells.

In contrast, a protumoural immune response results by inhibitory signals delivered by CTLA-4, TIM-3 and PD-1 as a consequence of T cell exhaustion. Also, FOXP3+ Treg cells play a critical role during the selection of high-avidity CD8 + T cells, reducing their functionality. Tumour cells can secrete cytokines and chemokines (e.g., TGF-β, CCL2) that recruit and stimulate suppressive cells such as Tregs, MDSCs, and M2 macrophages. M2 macrophages and MDSCs inhibit T-cell responses through nutrient sequestration via arginase, ROS, and NOS generation, as well as interference with trafficking into the tumour site. Tregs exert their inhibitory action on APCs, $CD8^+$ T cells, NKs, and $CD4^+$ Th1 T cells.

From Dushyanthen S. BMC Med. 2015

Besides degrading ECM components, MMPs can also activate chemokines, cytokines, adhesion molecules, and growth factors, which contribute to tumour progression by increasing tumour cell proliferation (e.g. the release of IGF from ECM by MMP3 and -7) or by promoting angiogenesis (e.g., activation of angiogenic factors by MMP1, -2, -9, and -14) (Chabottaux and Noel 2007).

In addition, abnormal physical characteristics of transformed breast tissue, such as irregular collagen cross-linking that results in ECM stiffening, contribute to tumour progression. In this context, a central role is played by lysyl oxidases (LOX), amine oxidases commonly expressed in breast cancer, which promote collagen cross-linking and enhances ECM stiffening. Accordingly, their inhibition in the MMTV-Neu model of BC increases tumour latency and decreases tumour burden (Levental, Yu et al. 2009). Moreover, the elevated expression of lysyl oxidase-like 2 is associated with worse prognosis in early stage ER-negative BCs (Barker, Chang et al. 2011).

1.1.4 Gene expression data in breast cancer

Although the current well-established clinical, histological and biological factors show strong association with prognosis, there is increasing evidence that these variables would not be sufficient to tailor the therapy to individual patients. The introduction of high-throughput technologies, coupled with powerful analytic tools, has redefined the classification of BC into biologically and clinically different groups based on gene expression patterns (Sorlie, Tibshirani et al. 2003, Sotiriou, Neo et al. 2003) and DNA copy alterations (Chin, DeVries et al. 2006).

Perou (Perou, Sorlie et al. 2000) and Sorlie (Sorlie, Perou et al. 2001) identified the so-called intrinsic subtype of BC, namely luminal A, luminal B, HER2-enriched, basal-like and normal breast-like. These subsets are associated with different risk to relapse and to response to therapy (Ribelles, Perez-Villa et al. 2013, Prat, Fan et al. 2015, Prat, Pineda et al. 2015) (Figure 5).

Subsets defined with histological criteria share similarities with intrinsic subtypes even though they are not identical. In particular, the molecular subtypes generally categorize with the receptor status. The ER+ broad group contains luminal A and luminal B tumours (ER+PR+HER2- and ER+PR+HER2+, respectively), which express luminal CK8/18 and genes associated with an active ER pathway. The ER-negative group includes at least 3 subtypes: normal breast-like, HER2 and basal-like. HER2-enriched tumours do not express hormonal receptors but overexpress HER2 and genes associated with the HER2 pathway. Basal-like tumours express genes usually found in normal basal/myoepithelial cells, including basal cytokeratins 5/17, nestin and EGFR. The HER2 and the basal-like subtypes have an aggressive clinical behavior. Most of basal-like tumours are triple negative breast cancers (TNBC) that do not express hormonal receptors or HER2 and account from 10-15% of all breast carcinomas. They represent an aggressive histological group, with very poor prognosis, as they do not respond to endocrine therapy or therapies targeting the HER2 receptor.

Although the Sørlie's intrinsic subtypes have set the standard for classification, several other molecular studies with varying numbers of genes included in the signature, allowed other classifications. For instance, Sotiriou et al. identified six

groups of BCs, which include three luminal-like, one HER2-enriched and two basal-like subtypes (Sotiriou, Neo et al. 2003).

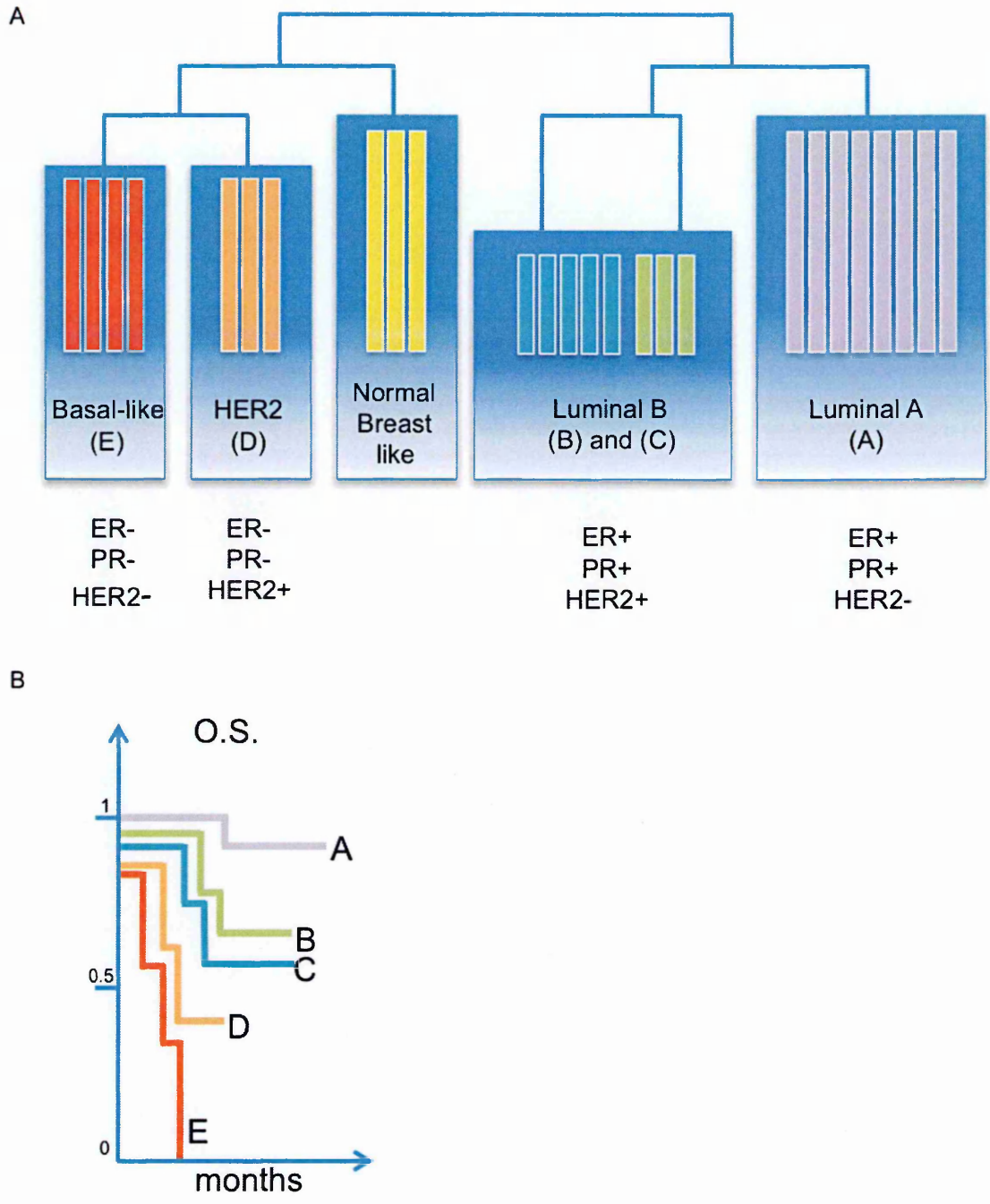

Figure 5 - Breast cancer intrinsic subtypes

A) Schematic view of breast cancer subtypes on the bases of gene expression patterns and relative presence of hormonal receptor in each subtype. B) Correlation of the intrinsic subtypes with the overall survival (OS) in human breast cancer patients.

Modified and adapted from from Sorlie et al. PNAS 2001 and Perou et al. Nature 2000

Fan et al. have suggested a 70-gene signature to classify tumours in four groups (Fan, Oh et al. 2006). Notably, Parker e al. reported a 50-gene classifier (PAM50), which mostly contains hormone receptor- and proliferation-related genes, and genes associated with myoepithelial and basal features. PAM50 signature has significant prognostic and predictive values in BC and is actually being applied in the clinical setting (Parker, Mullins et al. 2009, Gnant, Filipits et al. 2014) (Figure 6).

All these data have been obtained performing gene expression profiles in tumour specimens or cancer cells, but the well-established concept that tumour-associated stroma is activated by malignant epithelial cells to foster tumour growth, prompted several research groups to better understand the interactions between epithelial and stromal components of BC at the molecular level. Tumour microenvironment-related genes may indeed provide novel targets for diagnostic development and therapeutic intervention. Allinen and colleagues, performing the first systematic profiling of different tumour infiltrating stromal cells, demonstrated that gene expression alterations in all cell types within the tumour microenvironment go along with progression from normal breast tissue to DCIS and to IDC, providing evidence that these cell types participate in tumourigenesis (Allinen, Beroukhim et al. 2004). In particular, many of the genes involved in normal myoepithelial cell differentiation and function are downregulated in DCIS-associated myoepithelial cells, whereas genes that promote tumourigenesis are increased. Taking in the consideration this aspect, Ma and co-workers firstly discovered that most of the gene expression changes take place prior to local invasion to IDC (Ma, Salunga et al. 2003), and then extended the analysis to the

tumour stromal and demonstrated that, not only the tumour epithelium, but also the tumour stromal microenvironment undergoes extensive gene expression alterations during the transition from normal to pre-invasive stage of DCIS before IDC occurs (Ma, Dahiya et al. 2009).

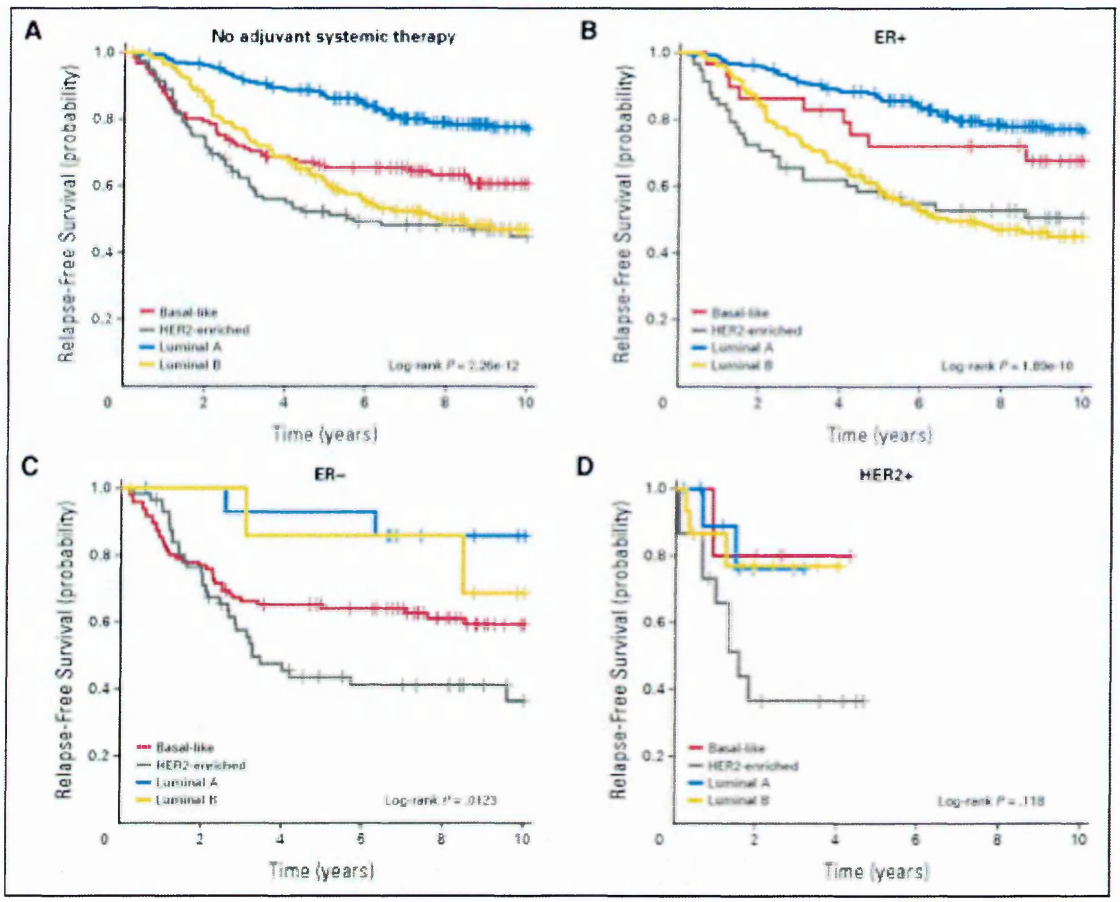

Figure 6 – Prognostic and predictive value of PAM50 signature.

Prognosis for relapse-free survival (RFS) of PAM50 intrinsic subtypes of breast cancer are described. (A) Outcome predictions according to Luminal A and B, HER2-enriched and basal-like subtypes in a test set of 710 patients with put lymphnode involvement and without systemic adjuvant therapy; (B) outcome by subtype in the same subset of patients with estrogen receptor (ER) –positive or negative (C) disease; (D) outcome by subtype in HER2-positive patients.

From Parker JS. J Clin Oncol. 2009

1.1.5 microRNAs

MicroRNAs or miRNAs are small (~22 nucleotides), evolutionary conserved, single-stranded, non-coding RNA molecules that bind mRNA targets to prevent their translation into proteins by distinct mechanisms. The biogenesis of microRNAs is tightly controlled in a step-by-step sequence of events and their deregulation is associated with many diseases, particularly cancer.

The majority of microRNA genes are transcribed by RNA polymerase II (Pol II) in the nucleus, where primary microRNAs (pri-microRNAs) are capped, spliced and polyadenylated (Lee, Kim et al. 2004). A pri-microRNAs can either give origin to a single microRNA or contain clusters of two or more microRNAs that are processed from a common primary transcript. The synthesis of mature microRNAs from pri-microRNAs occurs in two stages and it is catalyzed by two RNAse III-type proteins namely Drosha and Dicer. Drosha processes the pri-microRNA in the nucleus to a precursor microRNA (pre-microRNA) (Lee, Ahn et al. 2003), then transported to the cytoplasm by exportin-5 (XPO5) (Yi, Qin et al. 2003). In the cytoplasm, pre-microRNA are further processed to become mature microRNAs by Dicer (Park, Heo et al. 2011) and loaded on to the Argonaute (ago) protein to produce the effector RNA-induced silencing complex (RISC) (Kim, Han et al. 2009). The microRNA acts as a guide by base-pairing with target mRNA to negatively regulate its expression. The microRNA-mRNA interactions occur between the seed sequence of the microRNA, defined as the first 2-8 nucleotides from the 5' toward the 3', and the 3' untranslated region (3' UTR) of the mRNA.

The level of complementarity between the guide and the target mRNA determines which silencing mechanism will be employed: cleavage of target mRNA with

subsequent degradation or inhibition of its translation (Perron and Provost 2008) (Bartel 2004) (Figure 7).

Considering that a single microRNA can target hundreds to thousands different mRNAs, and that a mRNA can be coordinately suppressed by multiple different microRNAs, these small RNAs control almost every biological process (Krek, Grun et al. 2005).

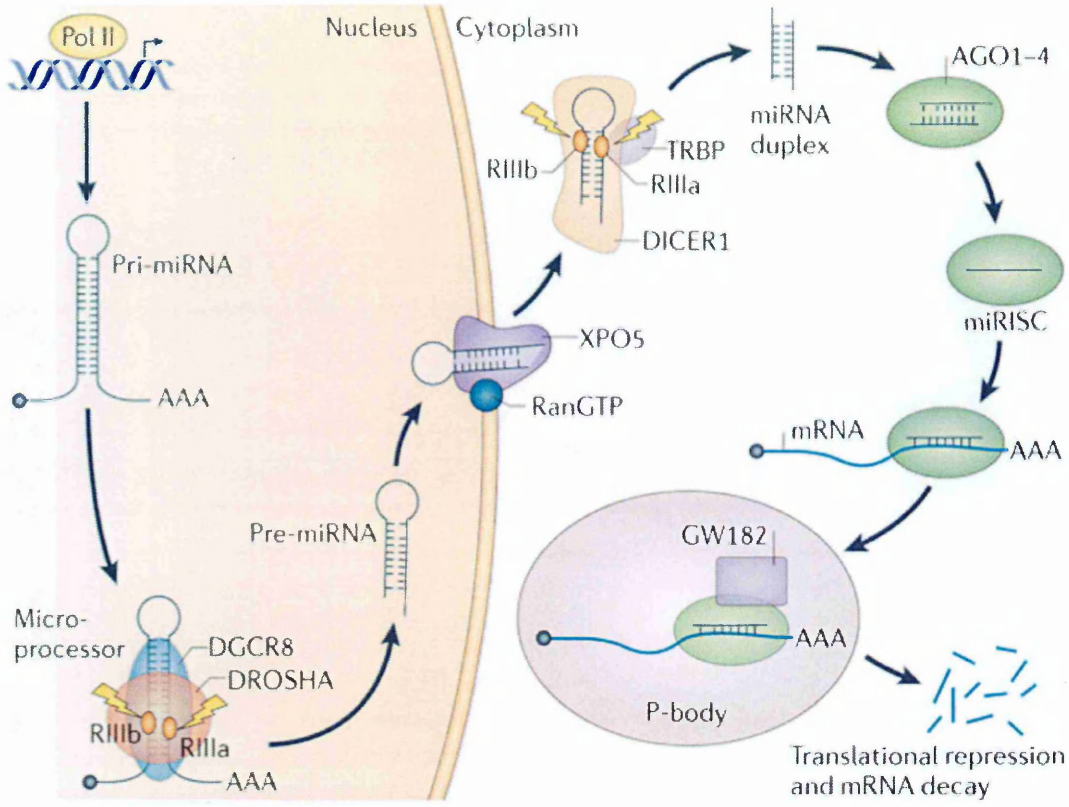

Figure 7 – microRNA biogenesis

MicroRNA (miRNA) genes are transcribed as primary miRNAs (pri-miRNAs) by RNA polymerase II (Pol II) in the nucleus. The long pri-miRNAs are cleaved by Microprocessor, which includes DROSHA and DiGeorge syndrome critical region 8 (DGCR8), to produce the 60–70-nucleotide precursor miRNAs (pre-miRNAs). The pre-miRNAs are then exported from the nucleus to the cytoplasm by exportin 5 (XPO5) and further processed by DICER1, a ribonuclease III (RIII) enzyme that produces the mature miRNAs. One strand of the mature miRNA (the guide strand) is loaded into the miRNA-induced silencing complex (miRISC), which contains DICER1 and Argonaute (AGO) proteins, directs the miRISC to target mRNAs by sequence complementary binding and mediates gene suppression by targeted mRNA degradation and translational repression in processing bodies (P-bodies). TRBP, transactivation-responsive RNA-binding protein.

From Lin and Gregory Nat Rev Cancer. 2015

1.1.6 Role of microRNAs in breast cancer

Recently, the definition of oncogenes and tumour suppressors has been expanded from the classical protein coding genes to include also microRNAs (Garzon, Fabbri et al. 2006, Wu, Sun et al. 2007), however their specific classification in these two categories can be difficult due to the complex expression patterns of microRNAs that differ from tissue to tissue (Lagos-Quintana, Rauhut et al. 2002, Lu, Getz et al. 2005, Lee and Dutta 2007, Mayr, Hemann et al. 2007). Indeed, it is not clear whether altered microRNA patterns could be considered a direct cause of cancer or rather and indirect effect of changes in the phenotype of the cells composing a transforming tissue. In addition, tissue specific expression could implicate that a single microRNA can act as a tumour suppressor in one context and as an oncogene (oncomiR) in another one.

What is now clear is that microRNAs may play roles in different steps of breast tumourigenesis, starting from tumour cell proliferation and differentiation to more advanced phases such as metastatic invasion (Goh, Loo et al. 2016).

Recent evidences have revealed that microRNAs are deregulated during cancer progression (McManus 2003, Meltzer 2005, Esquela-Kerscher and Slack 2006); accordingly, genome-wide studies demonstrated that microRNA genes are frequently located at cancer-associated genomic regions or in fragile sites. For example, downregulation of microRNAs is frequently observed in transformed tissue (Calin, Sevignani et al. 2004, Yanaihara, Caplen et al. 2006) suggesting a tumour suppressive role for down-regulated microRNAs in this setting. Nevertheless, because the abrogation of differentiation is considered a hallmark of cancer, the low-level expression of such microRNAs may be related to the state of

poor differentiation of cancer cells. If so, the expression of these microRNAs would be up-regulated when the cancer cells are induced to differentiate (Lu, Getz et al. 2005).

On the other side, up-regulation of microRNAs is frequently observed in different types of cancer, either haematological or solid tumours (Calin, Liu et al. 2004, Eis, Tam et al. 2005, Volinia, Calin et al. 2006). In BC, oncomiRs mostly affect proliferation of cancer cells and metastasis, and very few oncomiRs are known to be involved in angiogenesis and evasion of apoptosis (Figure 8).

Examples of breast oncomiRs are miR-10b, miR-21, miR-155, miR-373 and miR-520c (Figure 9). miR-10b targets homeobox 10 (HOXD10) a protein with a DNA binding domain, thereby promoting cell migration and invasion (Ma, Teruya-Feldstein et al. 2007, Tang, Ahmad et al. 2012). miR-21 has been reported to be associated with invasive and metastatic BC (Huang, Wu et al. 2009) mediating cell survival, proliferation, extravasation (Iorio, Ferracin et al. 2005) and regulating EMT and hypoxia-inducible factor 1-alpha (HIF1A) in BC stem-like cells (Han, Wang et al. 2012). Up-regulation of miR-155 has been shown to increase proliferation of the MCF7 breast cancer cell line (Zhang, Zhao et al. 2013). Finally, cell migration, invasion and metastases are associated with the expression of microRNA-373 and -520c, which act targeting the cell surface antigen CD44 involved in cell-cell adhesion (Huang, Gumireddy et al. 2008, Yan, Xu et al. 2011).

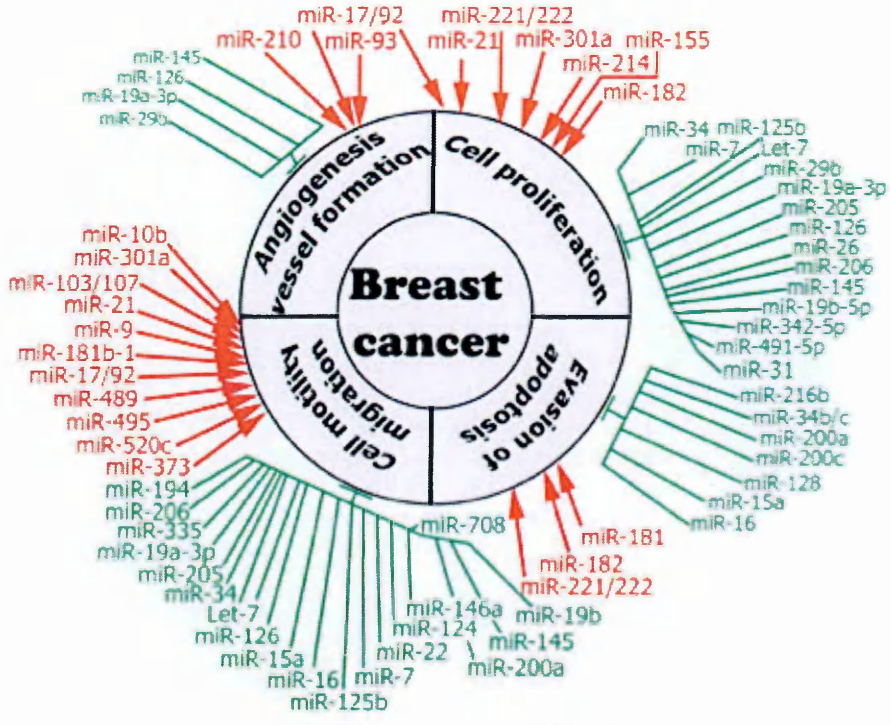

Figure 8 – Breast cancer-related microRNAs

Different miRNAs affecting the hallmarks of breast cancer. Oncogenic miRNAs and tumour suppressor miRNAs in breast cancer cells are shown in red and green, respectively

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Among tumour-suppressor microRNAs, miR-125b, miR-205, miR206 and miR-17-92 cluster have been show to have a role in BC. Specifically miR-125b targets erythropoietin as well as ERBB2 and is one of the most down-regulated microRNA in BC (Scott, Goga et al. 2007, Ferracin, Bassi et al. 2013); miR-205 is able to inhibit cell proliferation and promote apoptosis (Elgamal, Park et al. 2013, Liu, Mao et al. 2013); miR-206 inhibits cell proliferation, migration and invasion by targeting cyclin D2 (Li, Hong et al. 2013) and Jiang and colleagues revealed that the expression of miR-17-92 cluster in TNBC was associated with significantly reduced metastasis (Fan, Sethuraman et al. 2014, Jiang, Wang et al. 2014) (Figure 9).

These results have been obtained thanks to new advances in technology that have permitted to improve the accuracy, specificity and feasibility of microRNA profiling, from glass slide microarrays (Babak, Zhang et al. 2004, Sun, Koo et al. 2004) to bead-based flow cytometry and bead-arrays. All these techniques allowed the identification of microRNA expression patterns that characterize the presence of a state of disease, distinguishing between subtypes of normal and malignant tissues (Lu, Getz et al. 2005). MicroRNA signature profiles have been demonstrated to have a prognostic significance (Yanaihara, Caplen et al. 2006), to predict response to therapy (Meng, Henson et al. 2006) and treatment efficacy (Miller, Ghoshal et al. 2008), but also patient susceptibility to cancer and occurrence of metastasis (Calin, Ferracin et al. 2005, Ma, Teruya-Feldstein et al. 2007).

In addition, recent studies have found stable microRNAs not only in tumour tissues but also in readily available body fluids including serum, plasma, urine and saliva.

Indeed, a great effort has been put in the application of microRNAs as new circulating biomarkers for early diagnosis, prognosis or prediction of response to therapy in tumours (Wang and Chen 2014, Cheng 2015).

In the context of early diagnosis, a microRNA-based signature has been found to discriminate between early stage of BC and healthy controls (Kodahl, Lyng et al. 2014). Chan and colleagues (Chan, Liaw et al. 2013) identified four microRNAs (miR-1, miR-92a, miR133a and miR-133b) as significant diagnostic markers in serum samples of BC patients. Another group of four up-regulated microRNAs (miR-148b, miR-376c, miR409-3p and miR-801) in plasma of BC was found to be able to discriminate between stage I and II BC (Cuk, Zucknick et al. 2013). A prospective study identified three microRNAs significantly up-regulated only in serum of women who developed BC and not in healthy women (Godfrey, Xu et al. 2013) (Figure 10).

A

microRNA	Target	Function
miR-10b	HOXD10	Promotes cell migration, invasion and metastasis
miR-21	PDCD4, HIF1A	Promotes invasion, metastasis, migration and EMT
miR-155	SOCS1	Promotes cell growth and proliferation
	TP53INP1	Promotes proliferation
	FOXO3	Promotes proliferation and survival
miR-373	CD44	Promotes cell migration, invasion and metastasis
miR-520c	CD44	Promotes cell migration, invasion and metastasis

B

microRNA	Target	Function
miR-125b	EPO, EPOR, ERBB2	Inhibits cell proliferation, differentiation, migration and invasion
miR-205	HMGB3	Suppresses proliferation and invasion
mR-17-92	Mekk2	Promotes NK cell antitumoral activity and reduces metastasis
miR-206	Cyclin D2, Cx43	Reduces migration, invasion and metastasis
miR-200	ZEB1/2, SNAI1/2	Reduces tumor growth, metastasis and EMT
miR-146b	NFkB, STAT3	Reduces survival and metastasis
miR-126	IGFBP2, MERTK, PITPNC1	Reduces metastasis and angiogenesis
miR-335	SOX4, TNC	Suppresses metastasis and migration
miR-31	RhoA	Inhibits invasion and metastasis

Figure 9 – OncomiRs and tumour-suppressor microRNAs

A) List of major oncogenic microRNAs in breast cancer. EMT, epithelial-to-mesenchymal transition; FOXO3, forkhead box protein O3; HIF1A, hypoxia-inducible factor-1α; HOXD10, homeobox D10; miRNA, microRNA; PDCD4, programmed cell death protein 4; PTEN, phosphatase and tensin homolog; SOCS1, suppressor of cytokine signaling 1; TIMP3, metalloproteinase inhibitor 3; TM1, tropomyosine 1; TP53INP, tumour protein p53 inducible nuclear protein. B) List of major tumour suppressive microRNAs in breast cancer. CCNJ, cyclin J; CK2-α, casein kinase 2-alpha; Cx43, connexin 43; ENPEP, glutamylaminopeptidase or aminopeptidase A; EPO, erythropoietin; EPOR, erythropoietin receptor; ERBB2, Receptor tyrosine-protein kinase erbB-2 (human epidermal growth factor receptor 2); HMGB3, high-mobility group box 3 gene; IGFBP2, insulin-like growth factor-binding protein 2; MEGF9, multiple EGF-like domains 9; Mekk2, mitogen-activated protein kinase kinase kinase 2; MERTK, c-Mer tyrosine kinase; miRNA, microRNA; NFkB, nuclear factor kappa B; NK, natural killer; PITPNC1, phosphatidylinositol transfer protein, cytoplasmic 1; RhoA, Ras homolog gene family; SNAI1/2, snail family zinc finger 1/2; SOX4, SRY-related HMG-box 4; STAT3, signal transducer and activator of transcription 3; TNC, tenascin C; WAVE3, WAS protein family, member 3; ZEB1/2, zinc finger E-box binding homeobox ½

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microRNA	Expression (BC vs normal)	Sample type
miR-15	Upregulated	Serum
miR-18a	Upregulated	Serum
miR-107	Upregulated	Serum
miR-425	Upregulated	Serum
miR-133a	Downregulated	Serum
miR-139-5p	Downregulated	Serum
miR-143	Downregulated	Serum
miR-145	Downregulated	Serum
miR-365	Downregulated	Serum
miR-155	Upregulated	Serum
miR-1	Upregulated	Serum
miR-133a	Upregulated	Serum
miR-133b	Upregulated	Serum
miR-92a	Upregulated	Serum
miR-148b	Upregulated	Plasma
miR-376c	Upregulated	Plasma
miR-409-3p	Upregulated	Plasma
miR-801	Upregulated	Plasma
miR-16	Upregulated	Plasma
miR-21	Upregulated	Plasma
miR-451	Upregulated	Plasma
miR-145	Downregulated	Plasma
miR-18a	Overexpressed*	Serum
miR-181a	Overexpressed*	Serum
miR-222	Overexpressed*	Serum

Figure 10 – Circulating microRNAs in breast cancer patients

List of major diagnostic microRNA signatures for the early diagnosis of breast cancer.

From Eleni van Schooneveld Breast cancer research 2015

1.2 Mouse models of luminal breast cancer

Most of the knowledge about BC etiopathogenesis has been obtained taking advantage of mouse models spontaneously developing the disease. Although animal models reflect only in part the complex scenario of human diseases, they have deeply contributed in understanding the mechanisms driving BC pathogenesis.

As in BC patients the amplification of the HER2 gene and its overexpression at the messenger RNA or protein level occurs in about 20-30% of cases (Slamon, Clark et al. 1987), many of the most widely used animal models of breast carcinogenesis rely on the expression of HER2 in the mammary epithelium. In transgenic mouse models overexpressing HER2, mammary tissue transformation, in addition to the HER2 gene amplification, requires other genetic alterations (such as point mutations, deletions and insertions) (Siegel and Muller 1996), reflecting the clinical setting.

HER2, also known as ErbB2 or neu in rat, belongs to the epidermal growth factor receptor (EGFR) family, which also includes HER1 (EGFR, ErbB1), HER3 (ErbB3) and HER4 (ErbB4). HER2 is a 185-kd transmembrane receptor with an extracellular binding domain and a long cytoplasmic domain with tyrosine kinase activity. The extracellular domain is about 630 amino acids long and contains four subdomains arranged as a tandem repeat of a two-domain unit (Cho, Mason et al. 2003).

Although HER2 is the only receptor of the family without a known ligand, it is the preferred partner in heterodimer formation with other HER members, in particular HER3.

Binding of a ligand to the extracellular region of an EGFR family receptor induces its dimerization and activation of the cytoplasmic kinase, events that in turn lead to autophosphorylation and subsequent downstream signalling events. Intracellular signalling pathways activated include the mitogen-activated protein kinases (Ras/Raf/MEK/MAPK) pathway and the phosphoinositide 3-kinase (PI3K)–activated Akt (PI3K/Akt) pathway, responsible for cell proliferation and survival, respectively (Wieduwilt and Moasser 2008).

Her2 is expressed at low levels in normal tissues and it increases not only in breast but also in many other transformed tissues such as ovarian, prostate, gastric, colorectal, pancreatic, and lung (Hynes and Stern 1994).

In the MMTV-NeuT animal model, neoplastic transformation of the mammary epithelium occurs due either to the overexpression or activated form of rat HER2/neu (rHER2/neu), which transduces proliferative signals. rHER2/neu transgenic BALB/c females, NeuT mice, start to over express the product of the rHER-2/neu oncogene at early stages of mammary gland development and under hormone pressure during puberty. The tropism for the mammary epithelium is due to the presence of the mouse mammary tumour virus (MMTV) promoter, which allows the transformation only in this tissue with 100% penetrance. NeuT transgenic females develop mammary carcinomas with a well-defined progression, beginning at 3-5 weeks with a hyperplasia, condition in which epithelial cells start proliferating although the fat pads and the ductal structures are still evident. Then hyperplasia becomes atypical characterized by a proliferation of relatively uniform population of round epithelial cells assuming a stratified appearance with no formation of epithelial bridges. Starting with an occlusive growth of the epithelial

cells, dysplastic lesions degenerate into carcinoma *in situ* at around 15 weeks of age in which the ductal and acinar outlines remain distinct and separate from one another, with the persistence of intervening stroma. At nearly the 20th week alveolar groups of neoplastic cell with no myoepithelial lining infiltrate the surrounding adipose tissue. This feature is distinctive of invasive carcinoma. At around 30 weeks of age all mammary glands present palpable carcinoma (Quaglino, Mastini et al. 2008). Morphological examination of mammary neoplasia in NeuT mice indicates a lobular carcinoma of alveolar type very similar to human pathology, due to the preferential expression of HER2/neu products in the epithelium of lobular ducts and lobules. The transition form lobular hyperplasia to overt carcinoma is associated with a high epithelial proliferation rate and by activation of tumour angiogenic processes (Di Carlo, Diodoro et al. 1999).

An alternative splice form of the human HER2 gene, huHER2Δ16, has been detected in breast carcinomas and reported to comprise 4-9% of total HER2 transcripts (Kwong and Hung 1998). huHER2Δ16, which forms stable homodimers, has an increased transforming potency compared to wild-type HER2 form and its ectopic expression significantly enhanced *in vitro* the proliferation, migration and invasion of human MCF-7 breast tumour cells (Mitra, Brumlik et al. 2009). *In vivo* analyses showed that the huHER2Δ16 variant is tumourigenic *per se* and huHER2Δ16 mice, harbouring an in frame deletion of exon 16, develop multifocal mammary tumours with a rapid onset, higher incidence and shorter latency in comparison to MMTV-wtHER2 transgenic model (Castagnoli, Iezzi et al. 2014). Castiglioni et al. proposed a causal role of Δ16HER-2 in cancer development suggesting that malignant transformation occurs once the proportion

of Δ16HER-2 splice variant expression reaches a specific threshold. Conversely, wild-type HER-2 is not considered sufficient to induce transformation (Castiglioni, Tagliabue et al. 2006), suggesting that the huHER2Δ16 splice variant represents the transforming form of the HER2 oncoprotein (Marchini, Gabrielli et al. 2011).

Mitra et al. reported that 89% of patients with HER-2-positive breast tumours and disease progression to local lymph nodes, expressed Δ16HER2 (Mitra, Brumlik et al. 2009). Measurement of this variant may also be informative in predicting response to treatment with anti-HER-2 therapies Indeed, several studies have linked Δ16HER-2 with resistance to trastuzumab advocating the use of tyrosine kinase inhibitors as an alternative therapeutical approach (Castiglioni, Tagliabue et al. 2006, Mitra, Brumlik et al. 2009).

The Δ16HER2 variant activates multisignaling cascades including consistent phosphorylation of SRC kinase, which aberrant expression and activation occur in several tumour types and has been correlated with poor outcome (Guarino 2010). SRC is reported to be a driver of trastuzumab resistance and it is found to be highly expressed in primary breast tumours, with elevated levels of Δ16HER2 transcript, treated with trastuzumab-based regimens in adjuvant setting (Zhang, Huang et al. 2011). These tumours are initially dependent on HER2 and are responsive to trastuzumab and their progression leads to accumulation of genetic alterations that results in lower HER2 dependency and lower responsiveness to trastuzumab (Castagnoli, Iezzi et al. 2014). This lead to the idea that HER2 signaling-dependent tumours benefit from trastuzumab at early stages while in metastatic setting may escape from trastuzumab therapeutic effects.

Another widely used mouse model of mammary carcinogenesis is the polyoma

middle T (PyMT) model in which the tumour development is driven by the expression of polyoma virus middle T antigen under the control of MMTV promoter. Even though human breast tumour cells do not express the PyMT protein, the expression of this protein induces cancer development acting on several molecules such as Src, ras and PI3K that are often altered in human BC. Moreover, the expression of PyMT is associated with an increase in c-myc expression, a gene located in one of the three amplified chromosomal regions found in human primary BC, rendering the progression of PyMT model strictly similar to the women pathogenesis (Lin, Jones et al. 2003).

Premalignant lesions in PyMT mice involve the nipple-proximal terminal end bud located at the end of immature ducts surrounding the main collection duct and develop at early age, around 4-6 weeks of age. After prepubertal growth, new lesions arise in more distal locations, in a ER-dependent manner. Nevertheless, along with tumour progression, the distribution of focal atypical lesions along the ducts displays a gradient, with more numerous and larger lesions closer to the nipple than the other end of the ducts (Maglione, Moghanaki et al. 2001). Indeed, the nipple-proximal lesion is the area of origin of the tumour, developing in four principal stages similar to human tumours, namely hyperplasia, adenoma/mammary intraepithelial neoplasia (MIN), carcinoma in situ, and invasive carcinoma.

With the occurrence of advanced premalignant lesions, namely adenomas or mammary intraepithelial neoplasia (MIN), the single layer of myoepithelial cell starts to be incomplete in many points, only the laminin-1 expressing basement membrane remains intact. These preneoplastic lesions are characterized by

proliferation in the lumen of cells with cytological defects in the nucleus morphology.

At later time points, the phases of early and late carcinoma, laminin-1 start to be expressed in a scattered manner because of the loss of the acinar structure of the ducts and of the myoepithelial layer. An increase in vessels density also favours the infiltration of the tumour by immune cells (Lin, Jones et al. 2003). Tumour progression is associated with the loss of hormonal receptors, ER and PR, expression, a feature that distinguish mice tumours from tumours developing in women, which in many cases retain hormonal receptors expression.

Moreover, cyclin D1 increases in its expression whereas integrin beta-1 increases at early stages to decline later on. MMTV-PyVMT mammary tumours are enriched in proteases, including the MMPs, which are important not only for tissue remodelling but also for metastatization that occurs predominantly to the lung in this model of carcinogenesis.

1.3 Bone marrow and haematopoiesis

The bone is not only a supporting scaffold for the organism, but it represents an organ capable of fine-tuning immunity as it contains, within its cavity, the bone marrow (BM), which is the major site of haematopoiesis and bone formation.

Haematopoiesis is the process by which all mature blood cells are produced. Blood development in vertebrates involves two waves of haematopoiesis: the primitive and the definitive wave (Galloway and Zon 2003). The primitive wave, which involves eryhtroid progenitor cells, occurs in the embryo and it is a transitory phenomenon. Definitive haematopoiesis, by contrast, occurs later in development

and involves hematopoietic stem cells (HSCs), which are multipotent and can give rise to all blood lineages of the adult organism. In vertebrates, HSCs are generated in a specific region of the developing embryo and they migrate to the fetal liver and then to the BM, which is the location for HSCs in adults (Cumano and Godin 2007).

BM comprises two principal components: the vascular-stromal area where multipotential non-hematopoietic progenitor cells are located, and the hematopoietic parenchyma, mainly composed of HSCs and uncommitted hematopoietic progenitors (Kopp, Avecilla et al. 2005).

Lambersten and Weiss proposed a model to subdivide the different areas in the BM and, starting from the bone surface, they defined the endosteal, subendosteal, central and perisinusoidal niches (Figure 11)(Lambertsen and Weiss 1984). Multipotential non-hematopoietic progenitor cells or mesenchymal stem cells (MSCs), residing in the BM stroma, are able to differentiate into different tissue of mesenchymal origin such as osteoblasts, endothelial cells, reticular cells, fibroblasts and adipocytes, and are assembled in an organized network to physically and biologically support haematopoiesis. For instance, osteoblasts, endothelial cells, MSCs and CXCL12-abundant reticular (CAR) cells produce several factors to support HSCs, including thrombopoietin (TPO), CXCL12, angiopoietin and stem cell factor (SCF) (Chute, Muramoto et al. 2006, Greenbaum, Hsu et al. 2013). Endothelial cells are also responsible for the migration/extravasation of leukocytes into and out of the BM. Indeed, the microvasculature represents a physical and functional barrier from the peripheral circulation.

The hematopoietic parenchyma comprises HSCs and uncommitted hematopoietic progenitors, which colonize preferentially the endosteal and subendosteal niches. Conversely, committed progenitors and differentiated cells are distributed in the central and perisinusoidal niches, respectively (Bonomo, Monteiro et al. 2016). Quiescent HSCs are in close association with endosteal osteoblasts, but recent studies have suggest than only a small subpopulation of HSCs reside in this endosteal niche, whereas many HSCs are associated with the sinusoidal endothelium, which is referred to as the vascular niche (Kiel, Yilmaz et al. 2005, Kiel and Morrison 2006). As HSCs exit quiescence to enter a proliferative state, they migrate and colonize the subendosteal perivascular niche. Accordingly, maturation of cells in the BM follows a radial pattern, with progenitor cells residing close to the bone surface and differentiated cells in the inner part of BM parenchyma near central venous sinus (Figure 11).

BM regulates the functional maintenance of HSCs and immune cells through the presence of both hematopoietic and vascular niches, which are microenvironmental domains regulating proliferation, differentiation and quiescence. The regulation occurs either by direct cell-to-cell contact or through the secretion of growth factors, cytokines and components of the ECM (Mercier, Ragu et al. 2012).

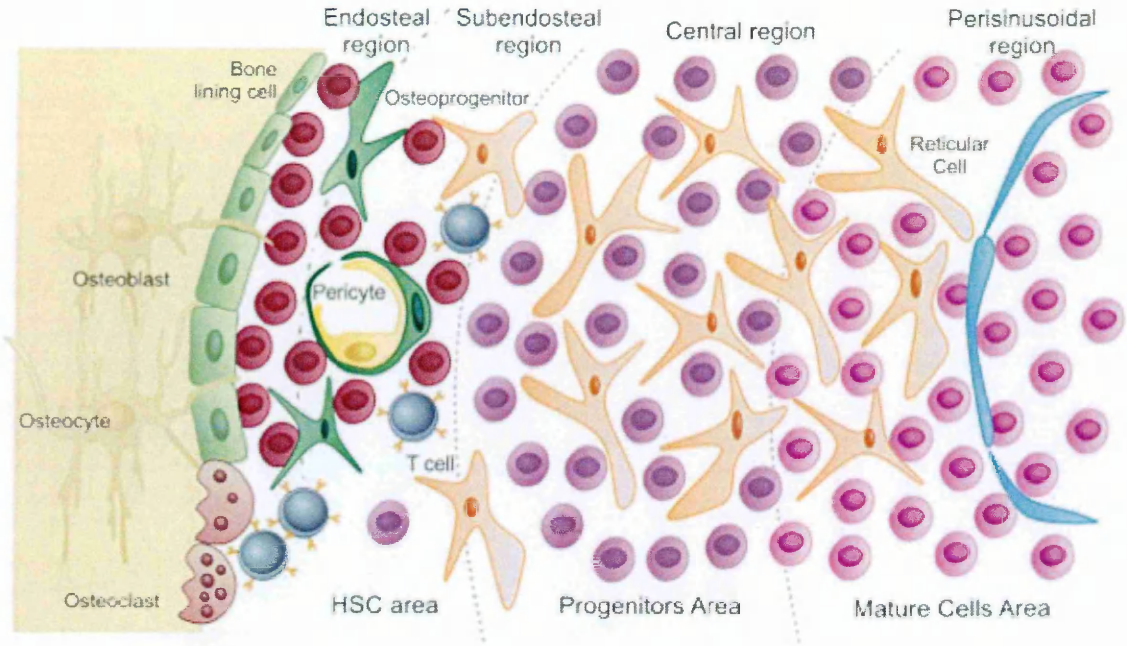


Figure 11 – Schematic representation of BM microenvironment under homeostatic conditions

HSC area, which harbors hematopoietic stem cells and uncommitted progenitors, comprises both endosteal and subendosteal niches. Committed progenitors and differentiated cells are distributed in the central and perisinusoidal niches, respectively. Quiescent hematopoietic stem cells are in close association with endosteal osteoblasts and bone-lining cells. As HSCs exit quiescence to proliferative states, they migrate and colonize the subendosteal perivascular niche, interacting with both endothelial cells and pericytes. Subendosteal sinusoid-derived pericytes serve as source for new osteoprogenitors, which will differentiate into osteoblasts during bone remodeling

Adapted from Journal of Cellular Biochemistry, 2014, Cordeiro-Spinetti et al.

Spangrude and colleagues were the first to demonstrate that HSCs were the only cells, in mouse BM, capable of fully reconstitute the entire hematopoietic system when transplanted into lethally irradiated mice (Spangrude, Heimfeld et al. 1988). After this study, mouse HSCs have been extensively purified and identified using additional cell-surface markers (Challen, Boles et al. 2009). HSCs include three multipotent populations: Long-Term (LT)-HSCs (c-Kit$^+$Lin-Sca-1$^+$CD34$^-$Flk2$^-$), Short-Term (ST)-HSCs (c-Kit$^+$Lin-Sca-1$^+$CD34$^+$Flk2$^-$), and Multi Potent Progenitors (MPPs, c-Kit$^+$Lin-Sca-1$^+$CD34$^+$Flk2$^+$).

LT-HSCs have self-renewal capability being able to maintain the stem cell pool or, alternatively, to differentiate into ST-HSCs or lineage-restricted progenitors that undergo extensive proliferation and differentiation to produce terminally differentiated hematopoietic cells. Immunophenotyping by flow cytometry (FC), using different cell surface markers, indicated that hematopoietic development has a hierarchical structure in which multi-potency is progressively restricted as cell specialization increases (Figure 12) (Seita and Weissman 2010).

HSCs initially give rise to the MPPs that no longer possess self-renewal ability, maintaining full-lineage differentiation potential, even if they are still heterogeneous. Further downstream, MPPs advance to oligo-potent progenitors: the common lymphoid progenitor (CLP, c-Kit$^+$Lin$^-$Sca-1$^+$) and the common myeloid progenitor (CMP, CD34$^+$CD16/32$^-$) (Kondo, Weissman et al. 1997, Akashi, Traver et al. 2000).

CLP gives rise to T and B lymphocytes, while CMP to megakaryocyte/erythroid progenitor (MEP, CD34$^-$CD16/32$^-$) and to granulocyte/macrophage progenitor (GMP, CD34$^+$CD16/32$^+$) (Nakorn, Miyamoto et al. 2003, Pronk, Rossi et al. 2007).

T and B lymphocytes, responsible for adaptive immunity, are distinguished by their site of differentiation. Mature T and B cells circulate between the blood and the peripheral lymphoid tissues and, after antigen recognition, B cells differentiate into antibody-secreting plasma cells, whereas T cells differentiate into effector T cells with a variety of functions. A third lineage of lymphoid-like cells, the natural killer (NK) cells, derive from the same progenitor but lack the antigen-specificity that is a hallmark of adaptive immune response.

Innate immune response is performed by leukocytes that derived from GMP such neutrophils, basophils and eosinophils (collectively known as granulocytes), macrophages and dendritic cells (DCs), whereas MEP gives rise to megakaryocites and mature erythrocytes.

Although BM, together with thymus, is a primary lymphoid organ and lacks organized T- and B-cell areas, the structural and functional features of the BM resemble a secondary lymphoid organ since it contains follicle-like structures similar to lymph nodes and spleen (Di Rosa and Pabst 2005). Indeed, in addition to its role as a primary lymphoid organ through the support of lymphoid development, BM can act as a host for various mature lymphoid cells. In fact, it is suggested that HSCs niche serves as "hub" for optimal T cell maintenance (Sabbagh, Snell et al. 2007).

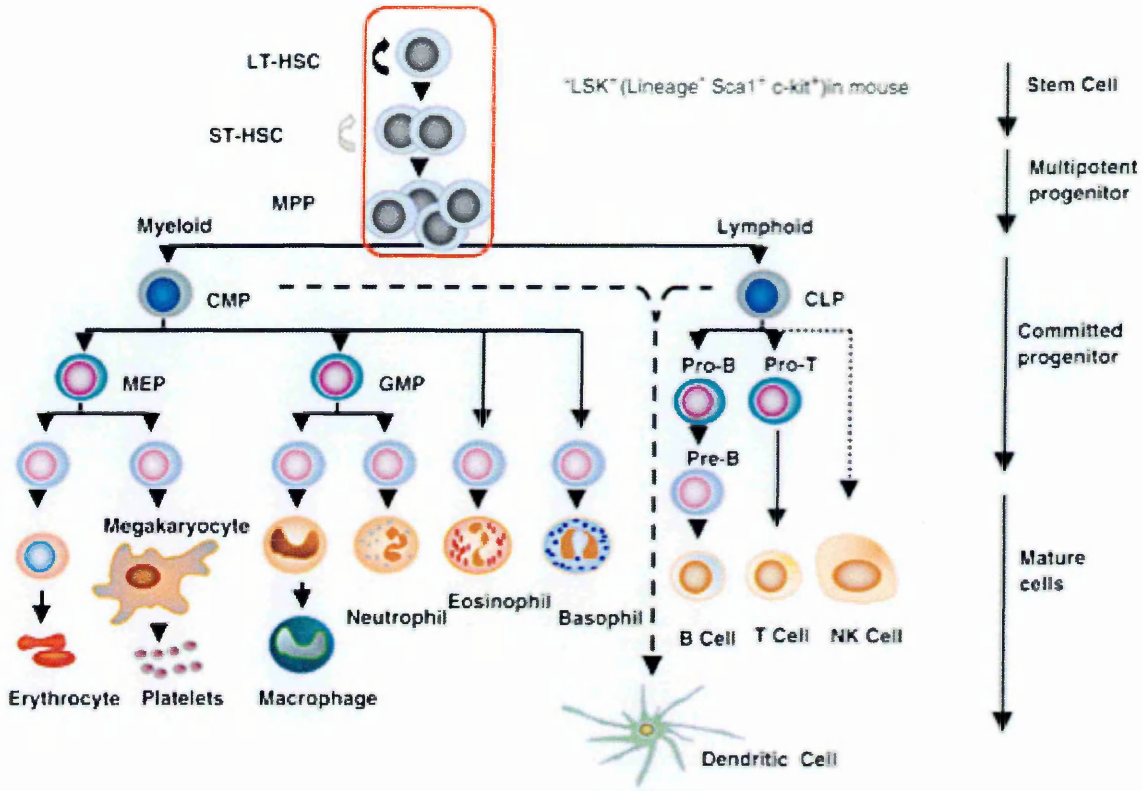


Figure 12 – The hierarchy of hematopoietic cells

LT-HSC, long-term repopulating HSC; ST-HSC, short term repopulating HSC; MPP, multipotent progenitor; CMP, common myeloid progenitor; CLP, common lymphoid progenitor; MEP, megakaryocyte/erythroid progenitor; GMP, granulocyte-macrophage progenitor. The encircled pluripotent population LT-HSC, ST-HSC and MPP are lin^-, $Sca\text{-}1^+$, $c\text{-}kit^+$.

From Larsson J. Oncogene 2005

1.3.1 Bone marrow-derived immune cells

- T cells

Mouse BM contains 1%-5% $CD3^+$ T cells (about 1.5% of $CD4^+$ and 2%-2.5% of $CD8^+$ T cells) (Price and Cerny 1999). Among $CD4^+$ cells approximately one-third are $CD4^+CD25^+$ regulatory T (Treg) cells (Zou, Barnett et al. 2004). Two-thirds of BM T cells express surface markers indicative of antigen experience, displaying a memory phenotype, such as high levels of CD44 (mouse-restricted marker) and CD122 (Mazo, Honczarenko et al. 2005). Treg cells can be mobilized from the BM by stimulation with granulocyte colony-stimulating factor (G-CSF) acting on CXCR4/CXCL12 signalling pathways (Zou, Barnett et al. 2004).

In addition, BM is the preferred site for proliferation of memory $CD8^+$ T cells, which are able to mount an effective secondary response (Becker, Coley et al. 2005). Memory $CD8^+$ T cells proliferate more extensively in the BM than they do in either secondary lymphoid or extra-lymphoid organs (Parretta, Cassese et al. 2005).

- B cells

As mentioned above, B cells develop and mature in BM before they egress into the peripheral blood. B cell development occurs preferentially in specific cellular niches associated with CXCL12- and IL-7- expressing cells and the transition from pre-pro-B cells to pro-B cells requires these signals. Recirculating memory B lymphocytes and active plasma cells secrete considerable levels of IL-6 and TGF-β, contributing to a myelopoietic balance (Tokoyoda, Egawa et al. 2004). $CD4^+$ T cells, expressing CXCR4, provide substantial assistance to differentiating B lymphocytes and produce cytokines that regulate myelopoiesis (Tokoyoda, Zehentmeier et al. 2009).

- NK cells (natural killer)

In normal adult mice, NK cells are found in thymus, bone marrow, liver and spleen and peripheral NK cells are rapidly deleted upon activation and replaced by NK cells that have been generated by *de novo* proliferation in the BM.

- Granulocytes

Collectively, basophils, eosinophils, and neutrophils are termed either granulocytes, because of the cytoplasmic granules that confer them a distinctive appearance in blood smears, or polymorphonuclear leukocytes, because of their irregularly shaped nuclei.

Eosinophils and basophils are recruited to sites of allergic inflammation and appear to be involved in the defense to parasites.

The protection from exogenous antigens and toxins is also exploited by mast cells that, arising from c-kit-expressing precursors in the BM, complete their maturation in tissues highly exposed to external environment.

Neutrophils are central effectors of innate immune responses because they are rapidly mobilized from the BM in case of infection and stress. The maintenance of neutrophils in the BM depends on CXCR4/CXCL12 signaling pathways (Eash, Means et al. 2009). Accordingly, the administration of G-CSF, which is reported to reduce the expression of CXCR4, mobilizes neutrophils from BM to peripheral circulation (Christopher, Liu et al. 2009).

- Dendritic cells (DC)

DCs play key role in both innate and adaptive immune responses. BM DCs are able to trigger central memory T cells-mediated responses and are essential for innate immunity to intracellular infection. Antigen presentation by BM DCs can

induce also the expansion of Treg cells and activate their ability to suppress cytokine secretion by effector T cells (Banchereau, Briere et al. 2000, Guermonprez, Valladeau et al. 2002).

- Myeloid-derived cells

Myeloid-derived cells are a heterogeneous population of cells consisting of myeloid progenitors, immature myeloid cells and macrophages. Immature myeloid cells are generated in the BM and differentiate into mature myeloid cells under physiological condition. Nevertheless, immature myeloid cells expanded and activated in tumour microenvironment, can acquire immune suppressive function. These myeloid-derived suppressor cells (MDSCs) exploit their immunosuppressive activity by producing immune suppressive factors such as arginase I (ARG1) or inducible nitric oxidase synthase (iNOS) or TGF-β (Gabrilovich and Nagaraj 2009). The nature and effector functions of MDSCs are described in details in chapter 1.3.3.

- Erythroid cells

In humans, erythropoiesis is confined to the BM, but in mice it also occurs in the spleen. The erythroid lineage derives from MEP, which can give rise to megakaryocyte and erythrocytes. The erythroid progenitor cells develop in two phases: erythroid burst-forming units (BFU-E) followed by the differentiation towards erythroid colony-forming unit-erythroid (CFU-e). This differentiation occurs upon stimulation with erythropoietin (EPO), which represents a critical factor for their maturation.

1.3.2 Extramedullary haematopoiesis

Haematopoiesis that occurs in organs other than BM, in response to pathological processes such as tumour growth, is termed extramedullary haematopoiesis (EH). There are two main types of EH, active and passive: the first occurs during both fetal development and immune response following an infection; the latter occurs because of failed BM haematopoiesis, in order to in part vicariate its function. Both phenomena produce antigen-presenting cells, granulocytes, NK cells, red blood cells and platelets.

EH refers to a broad range of hematopoietic activities form the early stages of lineage commitment. In fact, many BM-derived cells require further maturation in peripheral tissue in order to become functional immune cells. For instance, monocytes complete their maturation in peripheral tissue in order to become macrophages or DCs and B cells must be activated in the periphery to become memory and plasma cells, respectively.

In mice, BM haematopoiesis is delayed and becomes functionally active after birth. In the fetus, the yolk sac and the fetal liver provide the need for hematopoietic cells (Lux, Yoshimoto et al. 2008). After birth, the BM becomes the predominant site of haematopoiesis, together with an increasing number of progenitors colonizing the spleen, which remains a hematopoietic organ during all lifetime. In addition, the fetal liver acts as a site of haematopoiesis after birth, even if at low level. Together, liver and spleen are the main sites of EH after the activation of immune responses in the periphery (Figure 13).

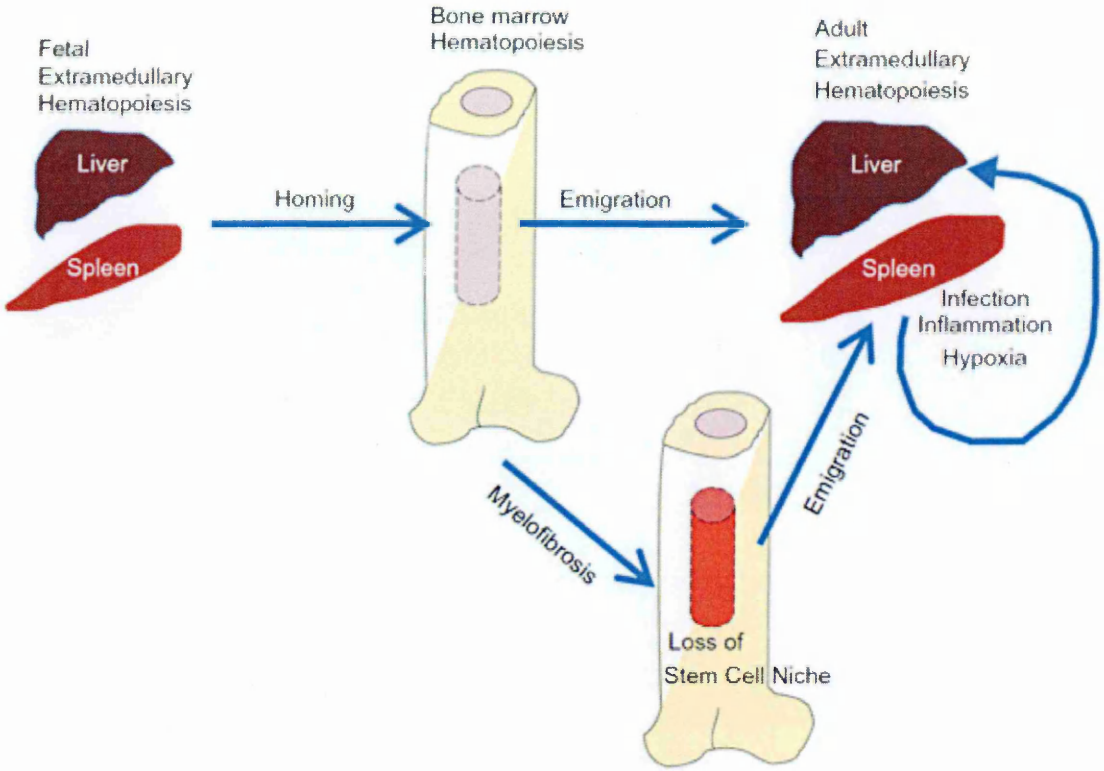


Figure 13 – Major forms of extramedullary haematopoiesis

Major forms of extramedullary haematopoiesis. Extramedullary haematopoiesis occurs early in fetal development and also plays important roles in adult life. Haematopoiesis occurs in the fetal liver and spleen. Hematopoietic stem and progenitor cells in the fetal liver migrate to the bone marrow and the marrow becomes the major hematopoietic site after birth. The hematopoietic stem and progenitor cells in the bone marrow emigrate to the periphery such as the liver and spleen. Upon infection and resultant immune responses, various hematopoietic factors including TLR ligands and cytokines promote extramedullary haematopoiesis in the liver and spleen. A major role of this extramedullary haematopoiesis is to produce functionally mature antigen-presenting cells and phagocytes. Excessive and prolonged extramedullary haematopoiesis in the periphery occurs in the presence of autoimmune diseases and chronic infection. In these situations, extramedullary haematopoiesis is harmful for the host. When malignant disease such as primary myelofibrosis occurs, the marrow becomes unsuitable to support haematopoiesis and extramedullary haematopoiesis is greatly increased

From Chang H Kim. J Blood Med. 2010

In particular, in mice, the spleen has predominantly lympho-erythropoietic function and its parenchyma contains a red pulp with eryhtropoietic activity and a lymphoid tissue called white pulp colonized by T and B cells. The spleen has three main function: i) it is able to elicit specific T or B lymphocytes-mediated immune reaction; ii) it serves as a reservoir of erythrocytes in mice; iii) it filters the blood removing old cells or infectious agents thanks to the presence of resident macrophages (Cesta 2006).

Microbial components such as Toll-like receptor (TLR) ligands (i.e. LPS) can directly activate haematopoiesis, increasing myeloid cell number in BM and spleen after LPS injection *in vivo*. Accordingly, infection may significant affect haematopoiesis both in BM and in peripheral organs, rendering TLR ligands effective regulators of EH (Nagai, Garrett et al. 2006).

The increase in myelopoiesis induced by EH during infection can lead to the production of MDSCs, similarly to what occurs in cancer settings.

While programmed EH is required to supplement hematopoietic activity in the BM, an excessive and disease-associated EH can occur and mediate chronic inflammation.

1.3.3 Myeloid-derived suppressor cells

Abnormal function and differentiation of myeloid cells, which result in a bidirectional molecular crosstalk between tumour and myeloid progenitor cells, is a hallmark of cancer. The accumulation of MDSCs, harbouring immunosuppressive function, is common in tumours, where they support progression through different

mechanisms such as promoting tumour cell survival, angiogenesis, invasion and metastasis formation (Condamine, Ramachandran et al. 2015).

Considering the phenotypic heterogeneity of this population the nomenclature of MDSCs remained debated until 2007, when MDSCs became the term for this cellular phenotype and function (Gabrilovich, Bronte et al. 2007). MDSCs are broadly defined as myeloid cells and represent a distinct population from terminally differentiated mature myeloid cells. Mouse MDSCs have been identified as CD11b+Gr1+ cells and, since Gr-1 is a combination of Ly6C and Ly6G markers, they can be accurately defined on the bases of these two molecules. $CD11b^{+}Ly6G^{low}Ly6C^{high}$ cells have a monocytic-like morphology, preferentially express iNOS, have an increased T cell suppressive activity and are identified as monocytic-MDSCs (Mo-MDSCs). In contrast, $CD11b^{+}Ly6G^{high}Ly6C^{low}$ cells have a granulocyte-like morphology, express high levels of ARG1, and are identified as granulocytic-MDSCs (G-MDSCs) (Figure 14). These cells have a polymorphonuclear (PMN) morphology and, therefore, the term PMN-MDSC is used interchangeably with the term G-MDSC (Youn, Nagaraj et al. 2008).

Mo-MDSCs and G-MDSCs present different mechanisms of immune suppression: Mo-MDSCs suppress both antigen-specific and non-specific T cell responses and have stronger suppressive activity than G-MDSCs; in contrast, G-MDSCs suppress only in an antigen-specific manner (Gabrilovich, Ostrand-Rosenberg et al. 2012). Several factors are implicated in MDSC-mediated immune suppression such as ARG1, iNOS, TGF-β, IL-10, COX2 and induction of Tregs (Huang, Pan et al. 2006, Youn, Nagaraj et al. 2008, Mao, Poschke et al. 2013).

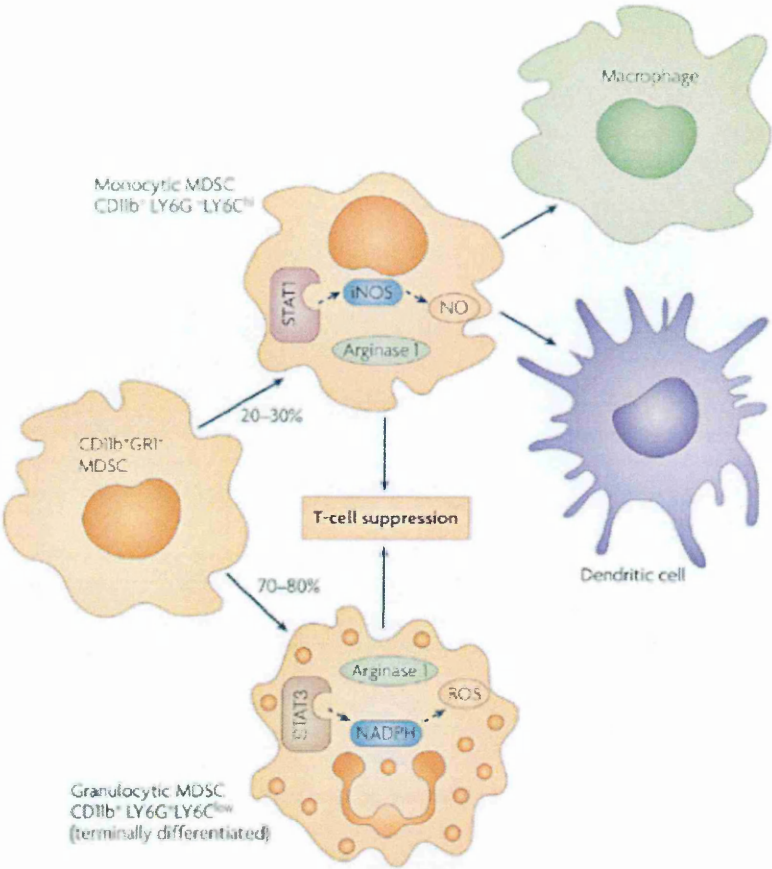


Figure 14 – Myeloid derived suppressor cells

Myeloid-derived suppressor cells (MDSCs) consist of two main subsets: monocytic MDSCs and granulocytic MDSCs. In most tumour models, Granulocytic subset of MDSCs has increased activity of signal transducer and activator of transcription 3 (STAT3) and NADPH, which results in high levels of reactive oxygen species (ROS) but low nitric oxide (NO) production. ROS induces the post-translational modification of T-cell receptors and may cause antigen-specific T-cell unresponsiveness. The monocytic MDSC subset has upregulated expression of STAT1 and inducible nitric oxide synthase (iNOS), and increased levels of NO but low ROS production. NO suppresses T-cell function through various different mechanisms that involve the inhibition of Janus kinase 3 and STAT5, the inhibition of MHC class II expression and the induction of T-cell apoptosis. Both subsets have increased levels of arginase 1, which causes T-cell suppression through depletion of L-arginine.

From Gabrilovich Nature Reviews Immunology 2009

Cells with a MDSC-like phenotype have a key role in tumour development and progression even via mechanisms that are not necessarily related to their ability to suppress tumour-specific immune response. Generally, the ability of MDSCs to support tumour growth and metastases can be divided into: i) protection of tumour cells from immune-mediated killing; ii) remodelling of the tumour microenvironment; iii) establishment of a pre-metastatic niche and, iv) interaction with tumour cells to induce proliferation and facilitate epithelial-to-mesenchymal transition (EMT).

In cancer, the accumulation of MDSCs requires two sets of signals. The first set supports their expansion and it is mediated mainly by GM-CSF, M-CSF, G-CSF and other growth factors produced by tumour cells and tumour stroma. Tumours that stimulate MDSC proliferation have been shown to secrete VEGF-A (Gabrilovich, Ishida et al. 1998, Melani, Chiodoni et al. 2003), GM-CSF (Bronte, Chappell et al. 1999), SCF (Pan, Wang et al. 2008), FMS-like tyrosine kinase 3 ligand (FLT3L) (Solheim, Reber et al. 2007), G-CSF (Waight, Hu et al. 2011) and M-CSF (Sawanobori, Ueha et al. 2008). Indeed, using transplantable tumour models, it has been shown that tumour-derived GM-CSF induces MDSC expansion and it is sufficient to generate immunosuppressive MDSCs *in vitro* (Bayne, Beatty et al. 2012).

Consistent with these studies, antibody neutralization or molecular inhibition of such secreted factors has been shown to reduce MDSC accumulation in the tumour microenvironment and to delay tumour development.

Although the expression of these factors is sufficient to expand MDSC, a second set of signals is required in order to confer them a suppressive phenotype. Several

pro-inflammatory cytokines such IL-6, TNF-α, IFN-γ and IL-1β are responsible for the induction of immunosuppressive capabilities (Gabrilovich, Ostrand-Rosenberg et al. 2012). Also, members of signal transducer and activator of transcription (STAT), such as STAT3, can regulate MDSC accumulation and suppressive functions acting on ARG1 production via C/EBPβ and IFN regulatory factor-8 (IRF8) (Waight, Netherby et al. 2013). Moreover, hypoxia-inducible factor 1 α (HIF1-α) promotes the suppressive activity of MDSCs via up-regulation of iNOS and ARG1 (Corzo, Condamine et al. 2010).

Recently, endoplasmic reticulum stress response, which protects cells form stress signals, has been shown to have a role in the immune suppression mediated by MDSCs (Mahadevan, Rodvold et al. 2011). For instance, MDSCs from tumour-bearing mice and cancer patients showed increased expression of DNA-damage-inducible transcript 3 (CHOP) and tumour MDSCs lacking *Chop* have decreased immune-regulatory functions and showed the ability to prime T cell function and induce antitumour responses (Thevenot, Sierra et al. 2014).

High mobility group box 1 (HMGB1) and peroxisome proliferator activated receptor gamma (PPARγ) have also been shown to contribute to immunosuppressive activity of MDSCs and have been recently added to the list of inflammatory mediators that control MDSC function (Wu, Yan et al. 2012, Parker, Sinha et al. 2014).

In several studies numbers of MDSCs correlate with poor prognosis, tumour vasculogenesis and tumour evasion of host immunity; a direct relationship between tumour burden and MDSC frequency has been demonstrated in several mouse tumour models (Donkor, Lahue et al. 2009, Younos, Dafferner et al. 2012)

and in clinical studies (Hickey 1991, Porembka, Mitchem et al. 2012, Wang, Chang et al. 2013).

Confirmation of these observations using transplantable tumours has been demonstrated in a FVB-NeuN model of mammary carcinogenesis (Abe, Dafferner et al. 2010), where a direct association between the development of metastatic mammary tumours and expansion of MDSCs subset has been shown.

G-MDSC subset is the predominant MDSC population in tumour-bearing mice, whereas Mo-MDSC frequency is increased in a limited number of tumour models. Studies using 4T1 tumour-bearing mice revealed a high frequency of G-MDSCs with a higher proliferation rate in in BM and spleen (Youn, Nagaraj et al. 2008).

Moreover, tumour-induced T cell-suppressive Ly6G+ myeloid cells are generated from an expanded stem and early progenitor compartment along with GMPs in BM of tumour-bearing mice (Casbon, Reynaud et al. 2015). In the same study the authors demonstrate that tumour production of G-CSF induces chronic activation of myeloid differentiation in the BM, which ultimately results in inefficient erythropoiesis, anemia, and enlarged spleens to meet the demands of neutrophil and red blood cells production during tumour progression.

MDSCs possess the ability to support tumour growth also through the remodeling of the tumour microenvironment producing VEGF and matrix-metalloproteinase-9 (MMP9), which are essential mediators of neoangiogenesis and tissue invasion, respectively (Tartour, Pere et al. 2011).

Tumour progression in BALB-NeuT mice is paralleled by the expansion of MDSCs and by the increase of VEGF and MMP-9 in the serum of tumour-bearing mice (Melani, Sangaletti et al. 2007). Indeed, tumour-derived VEGF is a key factor in the

expansion and function of MDSCs together with MMP9, which plays a central role in regulating the mobilization of HSCs from the BM (Heissig, Hattori et al. 2002). Mobilization from the BM is the first step of myeloid progenitor trafficking to inflammatory or tumour sites and it includes processes of alteration in cell-to-cell contact between myeloid progenitor and stromal cells.

In humans and mice, the treatment with selective antagonist of CXCR4 or CXCR4-blocking antibodies, results in a rapid mobilization of neutrophils, myeloid progenitors and MDSCs into the blood (Levesque, Hendy et al. 2003, Sawanobori, Ueha et al. 2008). This suggests that the disruption of CXCR4 signaling can contribute to G-CSF-induced mobilization and myelopoiesis. G-CSF administration also induces protease secretion in the BM microenvironment, thus contributing to mobilization (Levesque, Hendy et al. 2002). Accumulating evidences suggest that CXCL12 has a key role in controlling myeloid cell mobilization and homeostasis; in fact, CXCL12 or CXCR4 deficient mice die prenatally with neutrophilia and fail to establish BM haematopoiesis (Nagasawa, Hirota et al. 1996).

1.4 The role of Atf3 in cancer

As mentioned above, several lines of evidence have shaped the concept that tumourigenesis is a process regulated by a complex network of interactions between tumour cells, stromal cells, immune cells and the ECM, establishing a vicious cycle that fuels tumourigenesis.

In this scenario, considering the stress response as an early event in the neoplastic transformation, molecules involved in this process may contribute to cancer development and/or progression.

Activating transcription factor 3 (Atf3) is a immune transcription factor encoding for a member of the ATF/cyclic adenosine monophosphate response element-binding family of transcription factors, which share the basic region/leucine zipper DNA-binding motif and bind to the CREB/ATF and AP-1 consensus sequence (Hai and Hartman 2001).

Atf3 acts as a stress response protein, responding in normal cells to a plethora of insults such as endoplasmic reticulum stress, DNA damage, tissue injury and metabolic stress, through either induction or repression of different targets (Hai, Wolfgang et al. 1999).

For instance, when Atf3 is induced by TLR signaling, it inhibits the transcription of genes encoding pro-inflammatory mediators (Gilchrist, Thorsson et al. 2006). Transient TLR-driven signals induce NF-κB mediated-Atf3 transcription, which attenuates the expression of other NF-κB targets. Nevertheless, when TLR stimulation becomes persistent, the transcription of the NF-kB enhancer, C/EBP-δ, augments NF-κB signaling and overcomes Atf3-mediated inhibition (Litvak, Ramsey et al. 2009).

It has been shown that Atf3 is necessary to restrain the activation of the immune response in models of sepsis and in LPS-induced responses (Elizur, Adair-Kirk et al. 2007). Based on these findings Atf3 may be viewed as a "hub" of the cellular adaptive response network that helps cells to adapt to disturbance of homeostasis (Hai, Wolford et al. 2010).

Because of its activation in case of homeostasis alteration, the function of Atf3 has also been studied in cancer development and progression. Studies in cancer biology, highlighted Atf3 as a dichotomous protein, which may act as a tumour

suppressor, protecting normal and cancer cells from further transformation or as an oncogene, which is able to facilitate tumour progression, depending on the tumour type and its genetic portrait (Yin, Dewille et al. 2008, Thompson, Xu et al. 2009) (Figure 15).

The role of Atf3 as tumour suppressor is supported by pieces of evidence showing that the down-regulation of Atf3 in colon cancer promotes tumour growth and metastasis (Hackl, Lang et al. 2010) and its over-expression reduced the invasive potential of ovarian, bladder and lung cancer cells (Syed, Mukherjee et al. 2005, Jan, Tsai et al. 2012, Yuan, Yu et al. 2013).

However, its constitutive expression in a large portion of cancers suggest that, in many cases, Atf3 may exert oncogenic activities, directly on tumour cells or through its activity on the surrounding stroma. In fact, human BC samples showed a stromal cytoplasmatic expression pattern of Atf3 and Atf3 expression levels were significantly elevated in cancer-associated stroma from BC patients in comparison with normal stroma. In this setting, there was a correlation between CXCL12 and Atf3 as staining for CXCL12 was evident in ECM surrounding the Atf3-expressing cells (Buganim, Madar et al. 2011). Other studies have shown also that Atf3 might affect other members of the tumour stroma such as endothelial cells, smooth muscle cells and macrophages. In fact, Atf3 was shown to protect endothelial cells from TNFα-induced cell death (Kawauchi, Zhang et al. 2002) and the induction of Atf3 in these cells endowed them with the ability to form tubule structures (Okamoto, Iwamoto et al. 2006). In addition, Atf3 increases the survival and enhances the migration of vascular smooth muscle cells (Lv, Meng et al. 2011). These findings indicate that Atf3 might be involved in cancer progression through

the ability to promote the survival and formation of blood vessels in the vicinity of the tumour.

An oncogenic role for Atf3 has been shown in MCF10CA1 breast cancer epithelial cells since it is able to protect these cells from stress-induced cell cycle arrest, enhance their cell motility and EMT, thus increasing their tumour initiating features (Yin, Dewille et al. 2008, Yin, Wolford et al. 2010). Atf3 is found to be overexpressed in human breast tumours and, although the expression of Atf3 in breast cancer epithelial cells does not have clinical relevance, its expression in the stroma, specifically in mononuclear cells, correlates with worse outcome and with a higher proliferation rate assessed by Ki67 expression in the tumour. These findings highlighted an important role of Atf3 in mediating myeloid lineage responses in the TME and in favouring metastasis formation (Wolford, McConoughey et al. 2013). Accordingly, the macrophage/myeloid population is a key cell type in which Atf3 plays a functionally important role to facilitate metastasis. MMP9 is a direct target gene of ATF3 in macrophages and it is important for macrophages to enhance cancer cell invasiveness and transendothelial migration in vitro (Figure 16).

In PyMT mice, Atf3 expression in TAMs is induced by signals present in the TME and consistent with this observation, Atf3 is induced in BM-derived macrophages by IL-4, TGF-β hypoxia and co-culture with cancer cells (Wolford, McConoughey et al. 2013).

Although Atf3 function has been more extensively studied in macrophages and myeloid cells, it is able to mediate stress signals also in other cell types such as T cells, B cells, fibroblasts, adipocytes and endothelial cells.

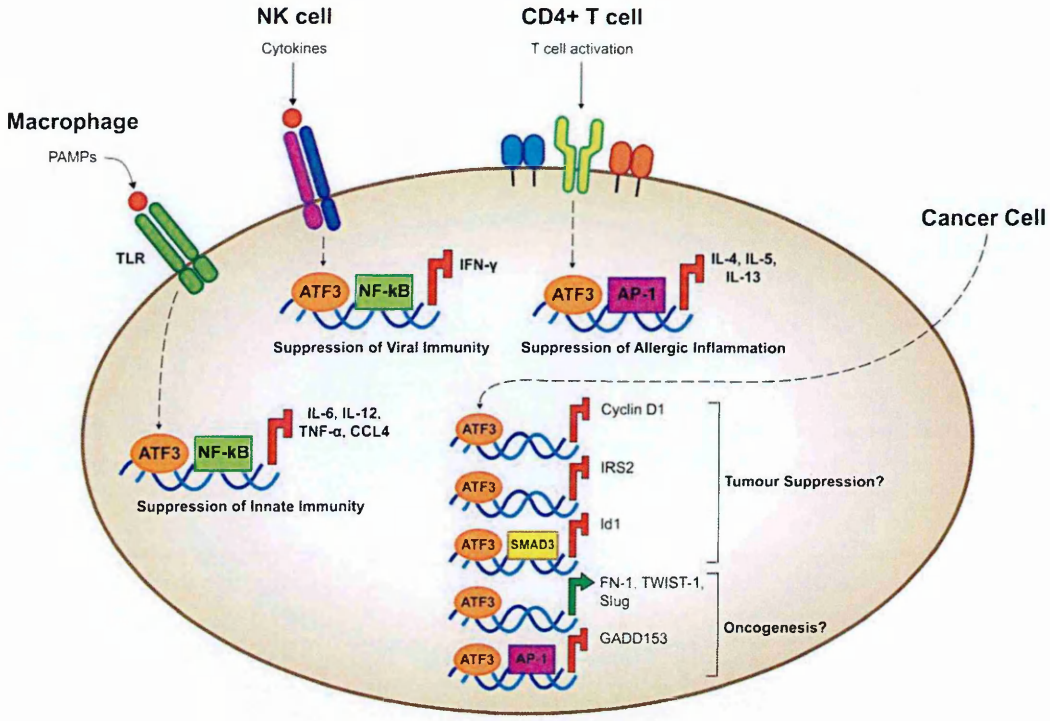


Figure 15 – Dichotomous role of Atf3

The role of ATF3 in host defense via the regulation of immune responses and cancer progression. In inactive immune cells, including macrophages, NK cells and $CD4^+$, ATF3 expression is maintained at low levels. Following activation of these cells by various signaling pathways, ATF3 is rapidly induced where it then binds its target promoters to regulate transcription. NF-κB and AP-1 are transcription factors, well known for their ability to promote transcription of inflammatory cytokines and chemokines. ATF3 appears to directly antagonise NF-κB and AP-1 driven promoters, resulting in decreased expression of distinct subsets of cytokines and chemokines. Overall, ATF3 is therefore able to limit the intensity of the inflammatory response to infection and allergy, and ultimately prevent pathological conditions associated with uncontrolled output of inflammatory mediators. Controversially, ATF3 has also been suggested to plays roles as both a tumour suppressor and oncogene. Although the mechanisms that regulate ATF3 induction in cancer cells are largely unclear, it is possible that ATF3 could prevent tumour growth by repressing transcription of cell cycle genes (such as cyclin D1 and Id1) and cell survival genes (such as IRS2). Conversely, induction of metastatic mediators FN-1, TWIST-1 and Slug, or suppression of GADD153, a known pro-apoptotic gene, may implicate ATF3 as an oncogene.

From Thompson M. J Mol Med 2009

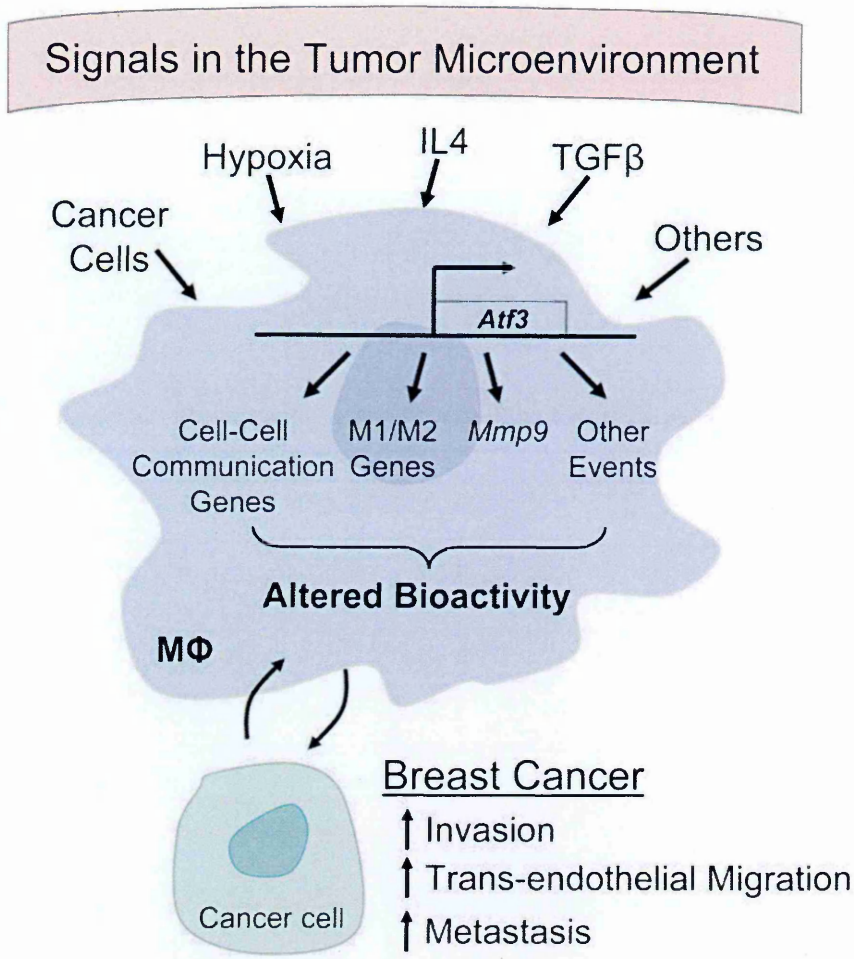


Figure 16 – Atf3 in macrophage

Model by which ATF3, a hub of the cellular adaptive response network, links various signals in the tumour microenvironment to alterations in macrophage transcriptional programs and bioactivities.

From Wolfrod CC JCI 2013

Taking in consideration all the aspects introduced so far, breast cancer features not only depend on the tissue where the transformation occurs but also on signals deriving from other organs. Since the tumour microenvironment is mainly composed of BM-derived immune cells, the importance of the BM in tumour development and progression is likely crucial. The immune system plays a key role in determining the fate of tumour cells, therefore the assessment of tumour-induced modification of haematopoiesis is relevant in understanding which variations in the BM are induced by tumour growth and which cell populations are involved in this process. The cross-communication between tumour cells and the surrounding environment is provided by several soluble factors, some of them circulating through the peripheral blood, being able to reach the BM parenchyma and tune, through local rearrangement, the quality of its response. Among the plethora of signals that are present in the circulation an important role is played by microRNAs, in light of their ability to modify the transcriptional program of several cells, also within the BM. As consequence, microRNAs may modulate the quality and the quantity of specific cell subsets that, once released from the BM, could reach the transforming tissue determining the fate of nascent tumours. The project described hereafter, focusing the attention on all the aspects that can modify the tumour development and assessing which signals may represent biomarkers of an incipient malignancy would offer a complete and precise picture of the modifications induced by the presence of a nascent tumour. Among them, it will be possible to identify those deserving more attention for an early diagnosis of neoplasia. Thanks to the recent advantages in microRNA and gene expression profile technology, the assessment of the transcriptional program of the BM and

the profile of microRNA both in BM and in the circulation, together with a deep immunephenotyping analysis, results in a combined approach in which all the modifications can be related to a specific stage of the disease.

2 Materials and Methods

2.1 Mice

Animals were maintained under pathogen free condition in filter-top cages at the animal facility of Fondazione IRCCS Istituto Nazionale dei Tumouri, Milan (authorization number: 240936/2005). All procedures involving animals were approved and performed in accordance with institutional guidelines and national law (D.lgs26/14), as well as the Declaration of Helsinki. BALB-NeuT female mice express the transforming rat oncogene c-erB2 (Her-2/neu) under the mouse mammary tumour virus (MMTV) promoter and spontaneously develop mammary carcinomas within a well-defined tumour progression time frame (Lucchini, Sacco et al. 1992, Boggio, Nicoletti et al. 1998). Female BALB/c mice were crossed with male NeuT mice and progeny was screened at 3 weeks of age for the presence of NeuT transgene by polymerase chain reaction (PCR). Her-2/neu-positive female were monitored weekly for mammary tumour development and progression, whereas non-transgenic littermates (wild type mice) served as age-matched controls.

PyMT mice (C57BL/6 genetic background), express the oncogene Polyoma Middle T antigen under the MMTV promoter and develop mammary tumours at around 15-18 weeks of age (Lin, Jones et al. 2003). PyMT transgenic female were crossed with male C57BL/6 wild type and progeny was screened at 3 weeks of age for the presence of the transgene by PCR. Female carrying the oncogene

were monitored weekly for mammary tumour development and progression, whereas non-transgenic littermates were used as age-matched controls

Similarly to BALB-NeuT, huHer2D16 mice (FVB genetic background) carry, under MMTV promoter, the human HER2 splice variant lacking exon-16 (Marchini, Gabrielli et al. 2011). The transgenic females developed multifocal mammary tumours with a rapid onset and an average latency of 15 weeks (Castagnoli, Iezzi et al. 2014). huHer2D16 mutated female, gently provided by Dr. Serenella Pupa, were crossed with male FVB wild type and progeny was screened at 3 weeks of age for the presence of the mutated allele by PCR. Female carrying the HER2 splice variant were monitored weekly for mammary tumour development and progression, wild type littermates were used as age-matched controls.

Separate groups of mice of each strain were sacrificed at:

- 6 or 9 and 12 weeks of age, time points that reflect early stages of tumour onset, in which tumour mass is not palpable yet, but tissue begin the transforming process;
- 24 weeks of age as representative of late time point when overt tumours are evident.

2.2 Collection of tissues, cells and surgery

Femurs and tibiae were collected bilaterally. Bone marrow (BM) cells were obtained by flushing femurs and tibiae with PBS 1X supplemented with 2% heat-inactivated fetal calf serum (FCS), with the use of a 22-gauge needle attached to a 1-mL syringe. 500 ul of blood was collected by intracardiac puncture with 20 ul of EDTA 0.5 M pH 8.0. 100-150 ul of blood was collected separately for flow

cytometry (FC) analysis and the remaining volume was processed for plasma separation within 2 hours. After surgical removal of the spleen, the organ was cut in two pieces: one piece was used as source of fresh cells; the other piece was used for histological analysis.

Fresh cells were obtained by mechanical disgregation of the organ in PBS 1X supplemented with 2% heat-inactivated FCS. Tissues for histological analysis were fixed in 10% neutral buffered formalin for almost 24 hours, washed in H_2O and stored in 80% EtOH before embedding in paraffin.

In addition to spleen, also mammary glands, contralateral femurs and tibias were collected for histological analysis.

In splenectomy experiments, spleens of 4 weeks NeuT and wt mice were surgically removed under aseptic conditions. Briefly, under anaesthesia with an intraperitoneal injection of ketamine (100 mg/Kg) and xylazine (5 mg/kg), a left flank incision was made to expose the spleen. The splenic blood vessels were cauterized using an electrocautery and the spleen removed by transecting the vessels just distal to the cauterized section. The peritoneum and the skin incisions were closed with wound clips, after checking for any haemorrhage in the abdominal cavity. Sham-operation was performed according to the splenectomy procedure but without removing the spleen.

2.3 Flow cytometry

Single-cell suspension preparations were treated with ACK lysis buffer (ammonium chloride potassium) on ice for 5 minutes to remove red blood cells. In order to avoid non-specific ligation of antibody to cells, each sample of

differentiated cells, BM, spleen and blood cells was Fc-blocked by CD16/32 antibody (eBioscience) before adding the specific antibodies. The following antibodies were used: Allophycocyanin-eFluor® 780 conjugated anti-mouse B220; PE-conjugated anti-mouse CD11b; FITC-conjugated anti-mouse F480, allophycocyanin conjugated anti-mouse Ly6C, PerCP-Cy5.5 conjugated anti-mouse Ly6G (Gr-1) and PE-Cy7 conjugated anti-mouse CD3 (all from eBioscience). For progenitors the following antibodies were used: PE-conjugated anti-mouse CD11b, CD11c, B220, Ly6G (Gr-1), Ter119, CD3, Pe-Cy7 conjugated anti-mouse CD117 (c-kit), FITC-conjugated anti-mouse CD34 and PerCP-Cy5.5 conjugated anti-mouse CD16/32 (all from eBioscience). For the evaluation of erythroid progenitors fresh blood cells were stained with FITC-conjugated anti-mouse CD71, PE-conjugated anti-mouse Ter119, PerCP-Cy5.5 conjugated anti-mouse CD11b, Pe-Cy7 conjugated anti-mouse CD117 (c-kit) and allophycocyanin-eFluor® 780 conjugated anti-mouse B220 (all from eBioscience). Antibodies were used at a final concentration of 5 µg/ml. Samples were acquired using a BD LSR II Fortessa instrument (Becton Dickinson) and analysed with FlowJo software (version 8.8.7, TreeStar). All samples were analysed in single; in each experiment at least 3 samples per group were analysed.

2.4 RNA extraction

Total RNA was extracted from BM cells using Trizol reagent (Life Technologies) following the manufacturer's instruction. RNA was treated with DNAse (Qiagen, Santa Clara, USA) and quantified by spectrophotometry (ND-2000c; NanoDrop Products, Wilmington, DE). Total RNA was extracted from plasma samples using

the commercial column-based system Qiagen miRNeasy® Mini Kit (Qiagen, Valencia, CA, USA), slightly modified by Exiqon (Vedbaek, Denmark) as described before (Callari, Tiberio et al. 2013). RNA integrity for microarray experiments was verified using the RNA 6000 Nano Kit (Agilent Technologies, Palo Alto, USA); all samples had an RNA integrity number (RIN) > 7, indicating good quality.

2.5 Gene expression and microRNA profiles

For gene expression profile (GEP), RNA was reverse transcribed, labeled with biotin and amplified overnight using the Illumina TotalPrep RNA Amplification Kit (Life Technologies). Biotinylated complementary RNA was hybridized to mouseWG-6 v2.0 Expression BeadChips (Illumina, San Diego, USA).

To assess the expression levels of microRNAs Illumina mouseWG-6 v2.0 microarrays designed on miRBase 19.0 from Agilent Technologies were used as described before (Callari, Tiberio et al. 2013).

For GEP profiling, Illumina mouseWG-6 v2.0 Expression BeadChips microarrays were scanned with Illumina BeadArray Reader, and raw data obtained using Illumina BeadStudio v3.3.8 were processed using the *lumi* package (Du, Kibbe et al. 2008) from R/Bioconductor (Huber, Carey et al. 2015). Raw data were log2-transformed, normalised using robust spline normalisation and filtered; only the probes with a detection p-value <0.01 in at least one sample were further analysed. Multiple probes representing the same gene were collapsed, and the probe with the highest detection rate was selected. In the case of equal detection rates, the probe with the greatest variation, as indicated by the interquartile range, was selected. For microRNA profiling, mouse miRNA miRBase 18.0 microarrays

from Agilent were scanned with an Agilent SureScan scanner, and raw data were collected using Agilent's Feature Extraction software v10.7. Raw data were pre-processed using an optimized version of the RMA algorithm implemented in the AgiMicroRna package (Lopez-Romero 2011). Data for bone marrow microRNA profiles were log2-transformed and normalised with quantile normalisation. For circulating microRNAs data were log2-transformed only, as standard normalisation strategies are not suitable for circulating microRNA data. microRNAs that were detected in at least 50% of samples, as indicated by the *gIsGeneDetected* parameter given by the Feature Extraction software, were kept for further analyses.

To merge gene expression data from different set of samples we applied ComBat to correct for batch effects.

For unsupervised analysis we merged gene expression data from the discovery and validation set to increase the number of samples. ComBat (Johnson, Li et al. 2007) was applied to remove the batch effect. Hierarchical clustering was applied with average linkage and 1-Pearson's correlation coefficient as distance metric.

2.6 Forced haemolysis

From an intracardiac puncture, using the same mouse, we collected 2 aliquots of plasma: one was maintained at 4° and the other one was centrifuged for 5 seconds at maximum speed in order to force an experimental-induced haemolysis. Both samples were considered haemolysed with a value > 0.2 at 414 nm of absorbance measured with spectrophotometer (ND-2000c; NanoDrop Products, Wilmington, DE).

2.7 Histology and immunohistochemistry

The BM and mammary glands were fixed with 10% neutral buffered formalin for 24 hours, washed with abundant H_2O and then paraffin embedded. Subsequently, four-micrometers-thick sections were cut and used for both morphological (haematoxylin & eosin, H&E) and immunohistochemistry (IHC) staining.

Before H&E and IHC staining tissue sections were deparaffinised and rehydrated. IHC was performed using a polymer detection method. (Novolink Polymer Detection Systems Novocastra Leica Biosystems Newcastle Ltd Product No: RE7280-K). The antigen unmasking technique was performed using Novocastra Epitope Retrieval Solutions pH 9 in PT Link Dako at 98°C for 30 minutes. Subsequently, the sections were brought to room temperature and washed in PBS. After neutralization of the endogenous peroxidase with 3% H_2O_2 and Fc blocking by a specific protein block the samples were incubated over night with the primary antibodies Mouse Monoclonal CXCL12/SDF-1(Clone #79018; 1:50 pH 6; R&D Systems), Rabbit Monoclonal CXCR4 (Clone UMB2; 1:100 pH 6; Epitomics), Mouse Monoclonal Nestin (Clone rat-401; 1:100 pH 9; Millipore); Rabbit Polyclonal ATF3 Activating transcription factor 3 (1:200 pH 6; Sigma-Aldrich) at 4 C°. Staining was revealed by polymer detection kit (Novocastra) and AEC (3-Amino-9-Ethylcarbazole) substrate chromogen. The slides were counterstained with Harris hematoxylin (Novocastra). Negative control stainings were performed by using mouse and rabbit immune sera instead of the primary antibodies. All the sections were analysed under a Zeiss AXIO Scope.A1 optical microscope (Zeiss Oberkochen Germany) and microphotographs were collected using a Zeiss Axiocam 503 Color.

2.8 Real-Time PCR analysis

For quantitative Real-Time PCR, RNA (0.1 μg) was reverse transcribed with the High Capacity cDNA Reverse Transcription Kit (Applied Biosystem) according to manufacturer's instructions. Real-Time PCR (RT-PCR) reactions were performed on ABI Prism 7900 HT (Applied Biosystem) using the TaqMan® Fast Universal PCR MasterMix (Applied Biosystem) according to manufacturer's instructions. Expression levels of each gene (Atf3, mmp9, Il-6) were normalised through the comparison with mouse Gapdh expression. Following probes have been used: Atf3 (Mm.PT.58.41654515 by IDT) ; mmp9: (Mm00442991_m1 by ThermoFisher Scientific), il-6 (Mm.PT.58.10005566 by IDT) and Gapdh (Mm.PT.39a.1b by IDT) Results were obtained using the comparative Ct method (Schmittgen and Livak 2008).

2.9 Western Blot analysis

Cells were lysed in a buffer containing 50 mM Tris HCl (pH 7.4); 150 mM NaCl; 1% Triton X-100; 0.1% SDS; 10 µg/ml Aprotinin (Sigma-Aldrich); 10 µg/ml Leupeptin (Sigma-Aldrich); 1 mM PMSF (Sigma) for 20 min at 4°C. The lysates were then centrifuged at 12,000 g for 10 min at 4°C and the supernatant collected. Lysates were subjected to Pierce™ BCA Protein assay kit (ThermoFisher) for total protein quantification before storing them at -80°C. After the determination of total proteins in samples, 20 µg for each sample were loaded on 10% or 4-12% Bis-Tris Gels (Novex, Invitrogen) and subjected to electrophoresis at constant 100 V in a X-Cell SureLock (Invitrogen) electrophoresis cell according to the manufacturer's instructions and using a SDS running buffer (0.025 M tris-base pH 8.8; 0.192 M

glycine; 0.1% SDS). Sodium Dodecyl Sulfate Poly-Acrylamide Gel Electrophoresis (SDS-PAGE) was performed as described in (Sambrook and Gething 1989) using a discontinuous buffer system (Laemmli 1970). This allows separation of proteins according to their relative molecular weight. The proteins were transferred from the gel to nitrocellulose blotting membranes (Amersham™ Protran™ 0.45 μm NC) previously activated in 100% methanol and transfer at 100 V for 1 hour. After electroblotting, the membranes were stained with Ponceau S to verify equal loading and transfer. Membranes were blocked in 5% low fat milk or BSA in TBS-T 1X for 1 hour then were incubated first with the primary antibody in TBS-T 5% milk or BSA for 1-12 hours depending on the antibody and after with a secondary antibody linked to the horse-radish peroxidase for 1 hour. The membranes were then washed and developed using enhanced chemiluminescence (ECL, Amersham). The following antibodies were used in these assays: anti-Atf3 1:200 (Santa Cruz, C-19, #sc-188) and anti-β-actin 1:2000 (Sigma, #A2006).

Membranes were exposed to autoradiography using high performance autoradiography film (Hyperfilm MP, Amersham Biosciences) and developed by an automatic developer (Curix 60, AGFA). For re-probing with different antibodies, membranes were incubated with a commercially available stripping buffer (Restore Western Blot Stripping Buffer, Pierce) for 45 minutes and stripping of the antibodies was checked by exposure of membranes to autoradiography as described above, before incubation with a different antibody.

2.10 Cell cultures

Human embryonic kidney (293T cells), mouse leukaemic monocyte macrophage RAW 264.7 (RAW) and mouse macrophage MT2 cell lines were cultured in DMEM (Gibco®) supplemented with 10% FCS, penicillin (100 U/mL), streptomycin (100mg/mL), non essential amino acids (NEEA)(Lonza), sodium pyruvate (1 μm)(Lonza) and N-2-hydroxyethylpiperazine-N-2-ethane sulfonic acid (HEPES) 0.01 M (Lonza). Mouse monocyte macrophage J774 cell line was cultured in RPMI 1640 (Lonza) supplemented as above described for DMEM.

2.11 Lentivirus production

Atf3-encoding lentiviral vector was constructed replacing the GFP sequence of pRRLsinCD68GFP.WprePGKNGFR, a myeloid specific lentiviral vector previously developed in our laboratory, with mouse mAtf3 coding sequence. A third-generation packaging system involving the transfection of four plasmids in the producer cells, namely two packaging plasmids (pMDLg/pRRE and pRSV-REV), an envelope plasmid (pMD2-VSV-G), and the lentiviral vector, was used. Lentiviral stocks were produced in 293T cells by Ca_3PO_4 co-transfection of the four plasmids. Twenty-four hours after transfection, the virus containing supernatant was harvested, passed through a 0.22 μm filter and purified by ultracentrifugation as described previously (De Palma and Naldini 2002). The viral stocks were stored at -80°c and the viral titer was assessed by expression of hNGFR (PE-conjugated anti-human) by FC in 293T cells infected with different dilution of virus. The presence of the truncated human NGFR reporter sequence allows an optimal

selection of cells after the infection. Infection of macrophage cell lines was performed at different multiplicity of infection (MOI), in presence of polybrene.

Lin^- cells isolated from wt mice were prestimulated overnight in Stemspan SFEM (STEMCELL Technologies) supplemented with 100 ng/mL SCF, 100 ng/mL Flt3L, 50 ng/mL TPO, 20 ng/mL IL3, and then lentiviral particles were added at a MOI 100. After 24 hours, cells were washed, counted and used for subsequent experiment (CFU assay). Infection efficiency was assessed by FC analysis evaluating the expression of the hNGFR. Infected cells were positively selected with Anti-Phycoerythrin (PE) MicroBeads (Milteny) after the incubation with α-CD271 (hNGFR, cat.557196 BD Pharmigen) following mchanufacturer's instruction. CFU assay was performed using Methocult (#3434 StemCell) following manufacturer's instruction.

2.12 In silico analysis

Gene Set Enrichment Analysis was carried out using GSEA (Subramanian, Tamayo et al. 2005) in pre-ranked mode using the c2_all collection form MSigDB database. Gene sets with a false discovery rate < 0.05 were considered statistically significant. Functional analysis of differentially expressed genes was carried out through Gene Ontology (biological processes domain). To estimate the relative amount of immune cells in the BM we applied CIBERSORT (Newman, Liu et al. 2015) to GEP data.

2.13 Statistical analysis

The analysis of data was performed using Prism software (GraphPad Software, Inc). Results are expressed as means ± SEM or SD. Statistical analysis was performed using a two-tailed Student's t-test with confidence intervals of 95%. Data were considered significantly different at $p<0.05$ ($^{*}p<0.05$, $^{**}p<0.01$, $^{***}p<0.005$ by two-tailed Student's t test). Differential expression analysis of microarray data was carried out using the limma package. P-values were corrected for multiple testing using the Benjamini-Hochberg false discovery rate (FDR) method. An FDR < 0.05 was considered statistically significant.

3 Aim of the study

The identification of early modifications induced by an incipient process of transformation is crucial to identify and treat cancers when they are still confined in a tissue. Considering the important role played by the immune system in tumour progression, the BM could be considered an important sensor of such phenomena. The aim of this work was to identify the variation in the BM that occurs concomitantly to an ongoing neoplastic transformation in a distant site. The hypothesis that BM is an early sensor of incipient neoplasia has been investigated taking advantage of animal models spontaneously developing mammary tumours. The use of mouse models offers the possibility to study the process of carcinogenesis starting from very early phases, an analysis difficult to be performed in the human setting.

The identification and characterisation of early changes occurring in the BM during tumour progression could offer the opportunity to identify key signals involved in the crosstalk between a developing tumour and the immune system, which could serve as early biomarkers of carcinogenesis.

In addition, the precise identification of those BM-centered modifications that are functionally relevant for the progression of nascent neoplasia could allow the discovery of novel potential therapeutic targets.

4 Results

4.1 Overt changes in bone marrow, spleen and peripheral blood populations are present in tumour-bearing mice and a trend of same modification characterizes mice with pre-cancerous lesions

It is well known that the immune system may play a crucial role during tumour development and progression mediating the process of immunesurveillance. In order to investigate whether and how an ongoing process of mammary carcinogenesis may impact on the hematopoietic compartment, BM, spleen and PBL of NeuT trangenic mice at different time points of tumour progression have been collected. A deep characterisation of their composition in terms of immune population has been performed at different stages of neoplastic progression taking advantage of different methodologies such as immunohistochemistry, FC and gene profiling. All the analyses have been carried out on specimens from the same mice in order to maximize the comparability of the results obtained by different methodologies.

Firstly, FC analyses have been performed to characterize the composition of differentiated and progenitors cells in BM and spleen, as well as differentiated cells circulating in the peripheral blood. A particular attention has been given to the myeloid compartment, since its role in tumour progression has already been shown in NeuT transgenic mice in which VEGF released by the tumour is the main

factor that conditions haematopoiesis toward the expansion of $CD11b^+/Gr1^+$ myeloid population (12750171).

The analysis has been firstly performed at late time points (24 weeks of age), when invasive carcinomas are fully developed. This served as positive reference for the subsequent analyses on earlier stages. Comparing differentiated cells in BM, spleen and PBL of tumour bearing NeuT mice, an increase of total $CD11b^+$ cells and of $CD11b^+Gr1^{high}Ly6C^{low}$ cells together with a reduction of $B220^+$ cells was observed in comparison to wt mice. This phenomenon of myeloid cell expansion and B cell contraction was observed not only in the BM but also in spleen and PBL of the same animals, suggesting a peripheral accrual of myeloid populations expanded in the BM (Figure 17A-C).

Considering that the cellular components in BM are strictly regulated, an expansion of a subset should reflect the contraction of another one, in order to maintain the same total amount of cells. For this reason the contraction of $B220^+$ B cells could simply be the consequence of the expansion of $CD11b^+$ myeloid cells. Nevertheless, the absolute number of $B220^+$ B cells in 24 weeks old NeuT mice was deeply contracted and not balanced by the enhanced number of $CD11b^+$ cells, likely suggesting that the observed B cell reduction was an independent phenomenon and not only a balance for $CD11b^+$ cell expansion (Figure 17D).

Also, the analysis of progenitor cells (GMP, CMP and MEP) in the BM of NeuT mice showed that at the late time point, MEP fraction was reduced while GMP subset was significantly expanded, confirming the boost in myelopoiesis observed in differentiated immune cell populations (Figure 17E). In addition, the spleen of

tumour-bearing mice showed a significant increase in lin^-ckit^+ cells, suggesting an increased extramedullary function of this organ (Figure 17F).

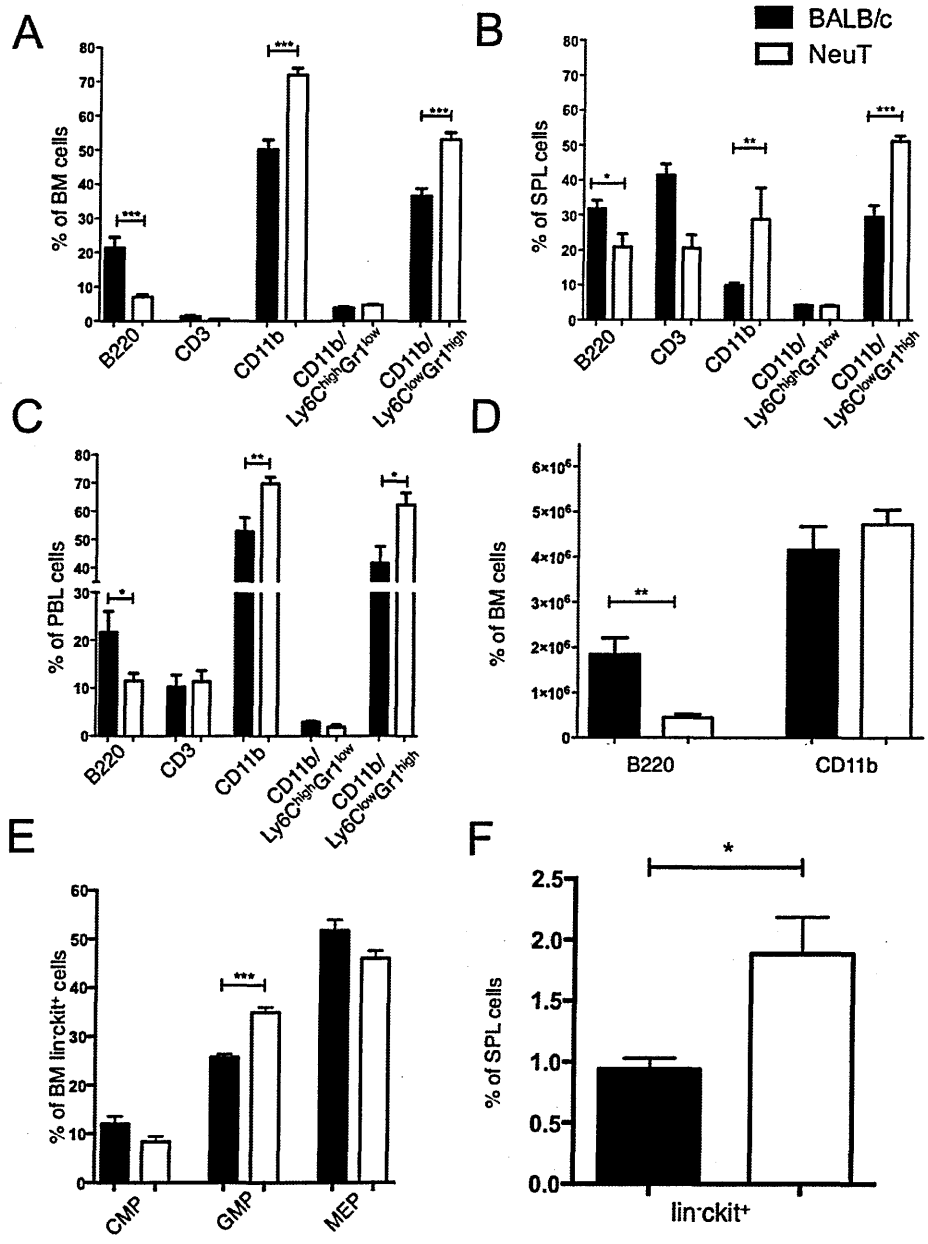


Figure 17 – Evaluation of the different leukocyte subsets in BM, spleen and PBL of tumour-bearing NeuT mice by flow cytometry

Analysis of differentiated cells in BM (A), spleen (B) and PBL (C) for $B220^+$ B cells, $CD3^+$ T cells, myeloid $CD11b^+$ cells and CD11bLyC/Gr1 subsets in BALB/c and NeuT mice at 24 weeks of age. D) Absolute number of $B220^+$ B cells and myeloid $CD11b^+$ cells in BM of BALB/c and NeuT mice at 24 weeks of age. E) Analysis of progenitors (CMP, GMP, MEP) in BM of BALB/c and NeuT mice at 24 weeks of age. F) Analysis of lin^-ckit^+ cells in SPL in BALB/c and NeuT mice at 24 weeks of age. NeuT (n=6), BALB/c (n=6).

In order to assess whether such alterations in the immune compartments associated to peripheral neoplasia were limited to the NeuT mammary model or could be more generally extended, the analysis of the abovementioned immune cell population was performed in two additional spontaneous breast cancer models, namely PyMT and huHer2Δ16 mice.

Considering the difference in the timing of tumour progression in PyMT and huHer2Δ16 mice, 22 and 24 weeks of age were selected, respectively, as representative of late time points.

Among differentiated cells, an accumulation of $CD11b^+$, $CD11b^+Gr1^{high}Ly6C^{low}$ cells and a reduction of $B220^+$ and $CD3^+$ cells in the BM of PyMT in comparison to healthy mice were found (Figure 18A). Consistently, this result suggested that the PyMT carcinogenesis is associated with myeloid granulocytic cell expansion in the BM, which is remarkable at advanced stages. These data are in agreement with the observations in NeuT tumour-bearing mice, at least for late stages of carcinogenesis.

On the other hand, the same analysis in the huHer2Δ16 model at late time points did not show any significant expansion of myeloid cells (Figure 18B).

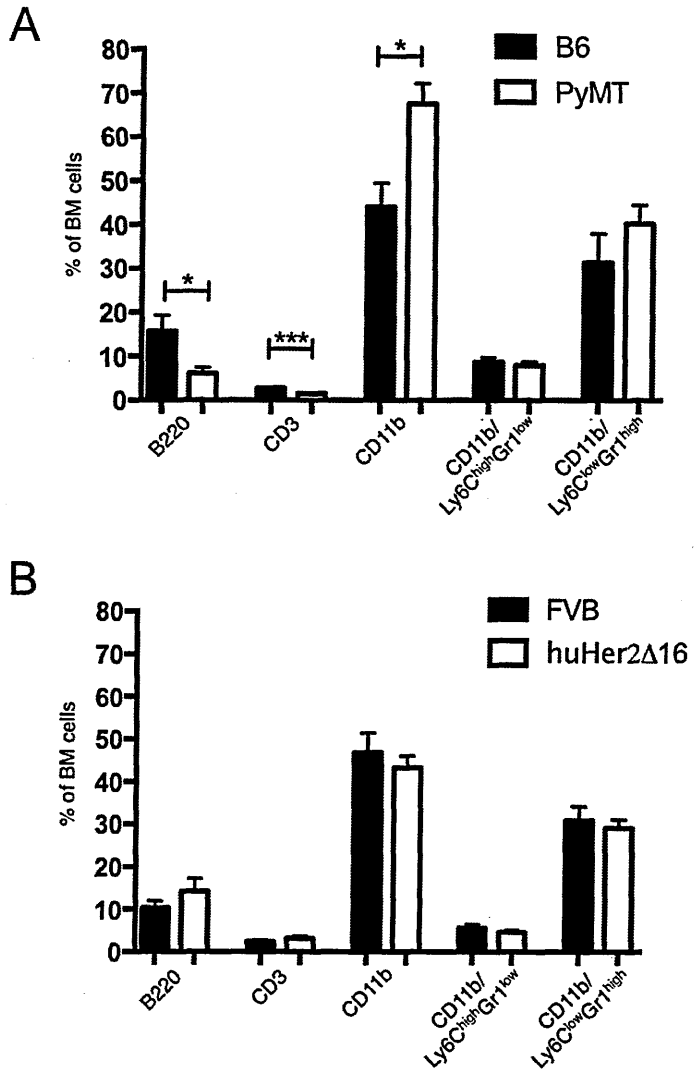


Figure 18 – Evaluation of the different leukocyte subsets in BM of tumour-bearing PyMT and huHer2Δ16 mice by flow cytometry

A) Analysis of differentiated cells in BM for B220$^+$ B cells, CD3$^+$ T cells, myeloid CD11b$^+$ cells and CD11bLyC/Gr1 subsets in B6 and PyMT mice at 22 weeks of age. PyMT (n=7); B6 (n=4).
B) Analysis of differentiated cells in BM for B220$^+$ B cells, CD3$^+$ T cells, myeloid CD11b$^+$ cells and CD11bLyC/Gr1 subsets in FVB and huHer2Δ16 mice at 24 weeks of age. huHer2Δ16 (n=13); FVB (n=7).

4.2 Slight modifications of immune cell populations characterize mice with pre-cancerous lesions

To assess whether the modifications in immune cell compartments observed at late time point could also be detected earlier, when no overt invasive carcinoma is present, the same analysis was carried out at 6 and 12 weeks of age, which correspond to hyperplasia and pre-cancerous *in situ* lesions, respectively.

FC analysis at 6 weeks did not show significant differences among differentiated cells in BM, spleen and PBL in NeuT mice compared with their wt counterpart (Figure 19A-C). In the spleen of NeuT mice at 12 weeks of age, an increased fraction of immature myeloid cells, belonging to granulocytic $CD11b^{+}Gr1^{high}Ly6C^{low}$ and monocytic $CD11b^{+}Gr1^{low}Ly6C^{high}$ compartments was found, in comparison to wt mice. In the circulation of the same mice, data were consistent with findings in the spleen. Indeed, an expansion of $CD11b^{+}Gr1^{high}Ly6C^{low}$ was observed in NeuT mice. Although not statistically significant, at the same time point, in the BM of NeuT mice there is a slight reduction of B cell compartment ($B220^{+}$ cells) and an increase of $CD11b^{+}$ cells in comparison to age-matched wt mice (Figure 19D-F).

The analysis of progenitor cells, during disease progression, did not show statistically significant difference neither in BM nor in spleen of NeuT in comparison to wt mice (not shown).

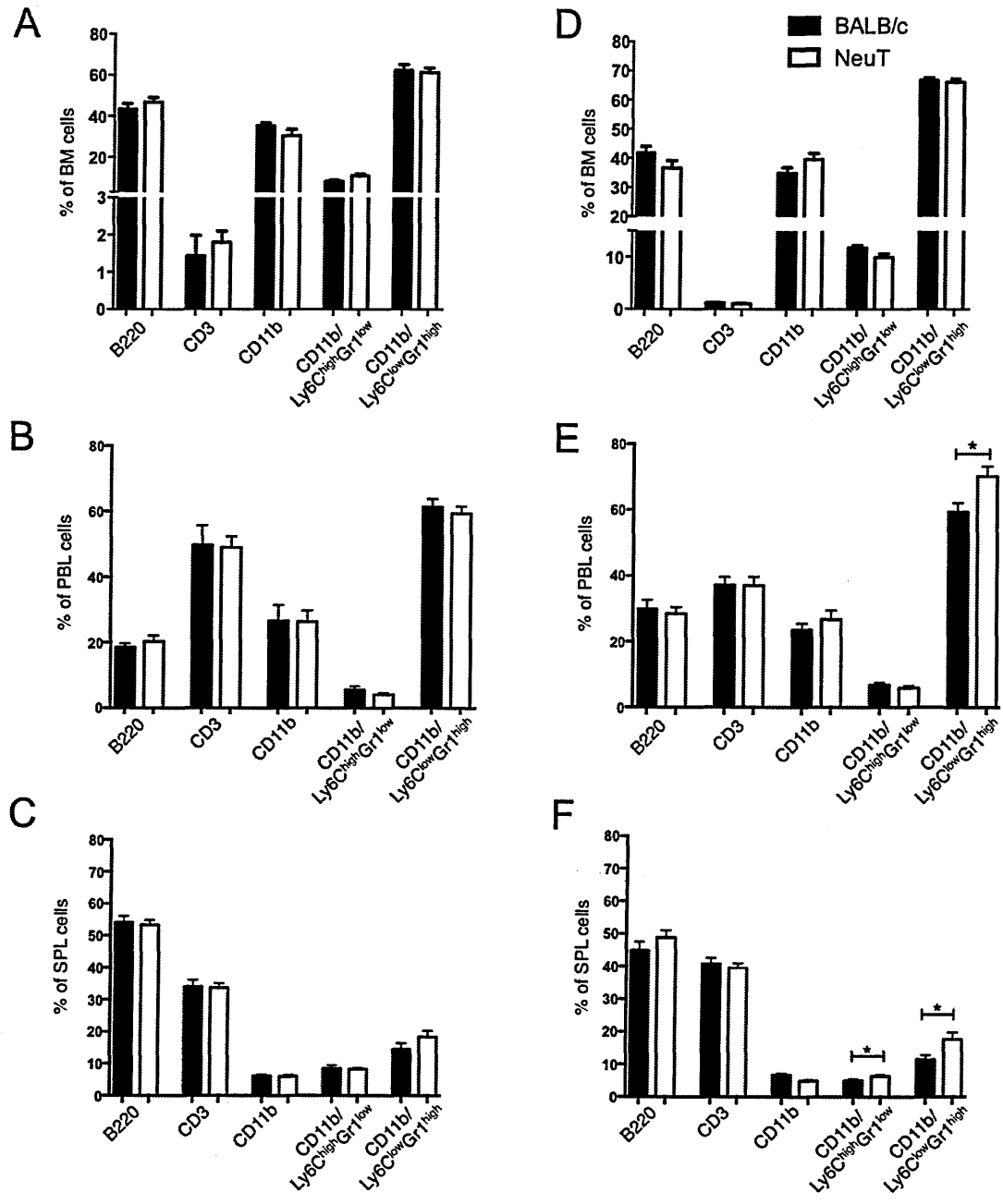


Figure 19 – Evaluation of the different leukocyte subsets in BM, spleen and PBL of 6 and 12 weeks old NeuT mice by flow cytometry

Analysis of differentiated cells at 6 weeks of age in BM (A), PBL (B) and spleen (C) for $B220^+$ B cells, $CD3^+$ T cells, myeloid $CD11b^+$ cells and CD11bLy6C/Gr1 subsets of BALB/c (n=11) and NeuT (n=12) mice.

Analysis of differentiated cells at 12 weeks of age in BM (A), PBL (B) and spleen (C) for $B220^+$ B cells, $CD3^+$ T cells, myeloid $CD11b^+$ cells and CD11bLyC/Gr1 subsets for BALB/c (n=10) and NeuT (n=10) mice.

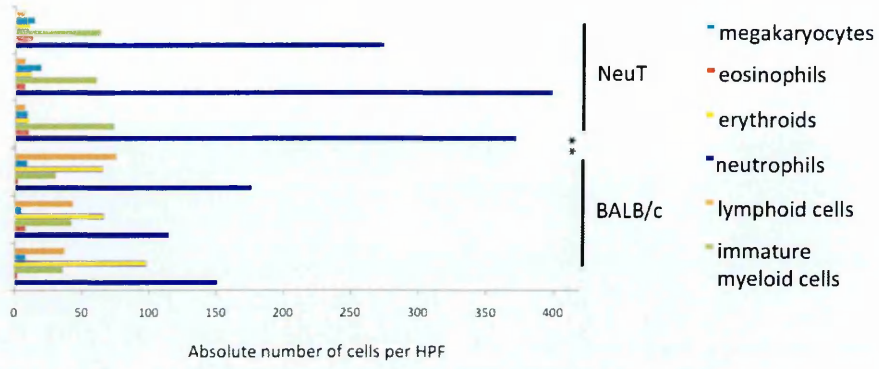
Considering the timing of breast cancer progression in PyMT and huHer2Δ16 models, 6-12 weeks and 6-9 weeks of age have been selected as representatives of early stages of progression, respectively. FC analysis on early samples did not detect any significant differences for both differentiated and progenitor cells in both mouse models (not shown).

4.3 Mice with both advanced and pre-cancerous lesions showed significant BM stroma rearrangements

To better characterize modification occurring in immune cell populations during breast cancer progression, histopathology analyses have also been performed by an expert pathologist, Prof. Claudio Tripodo from the University of Palermo.

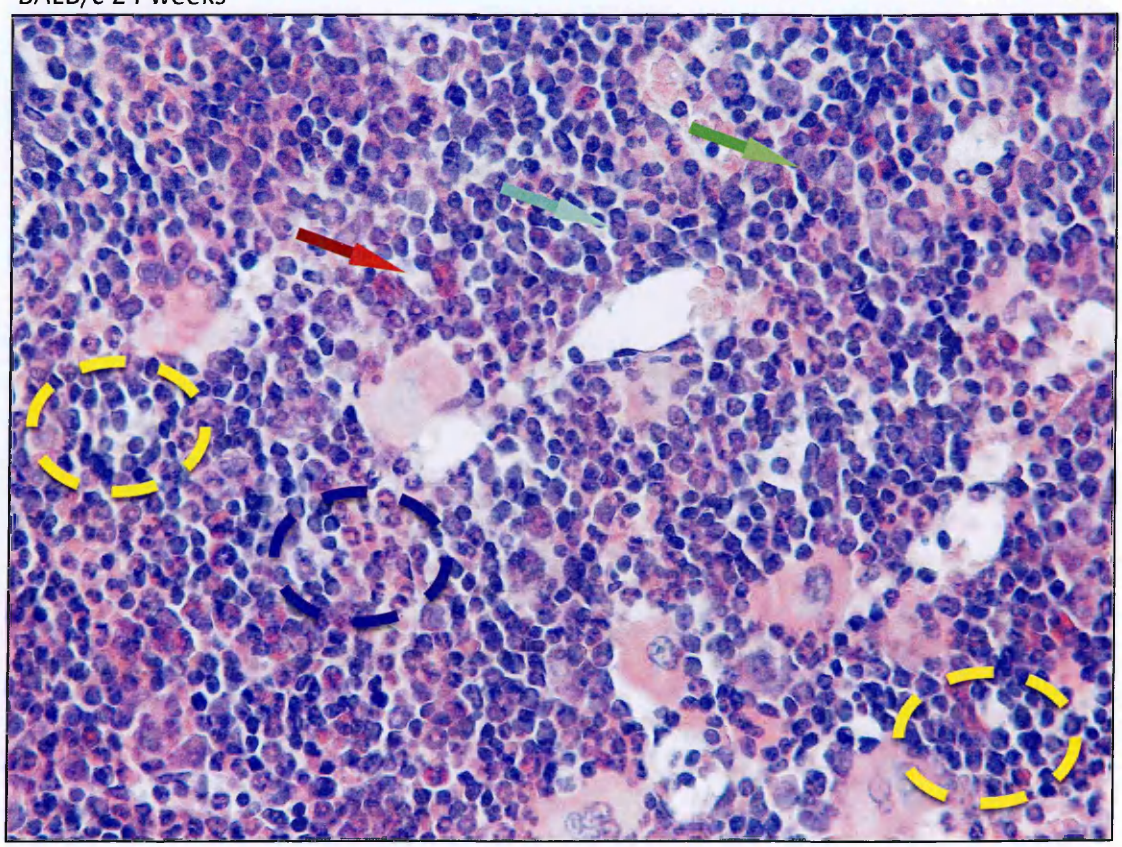
At late stage a profound modification in the composition and spatial arrangement of the hematopoietic populations between tumour-bearing and wt mice has been observed. In particular, as already highlighted by FC analysis, an increase of the density of myeloid granulocytic cells with immature morphology has been found in BM of tumour bearing mice. This expansion was also associated with a displacement of lymphoid and erythroid niches and in the contraction of the erythroid pool itself (Figure 20).

A



B

BALB/c 24 weeks



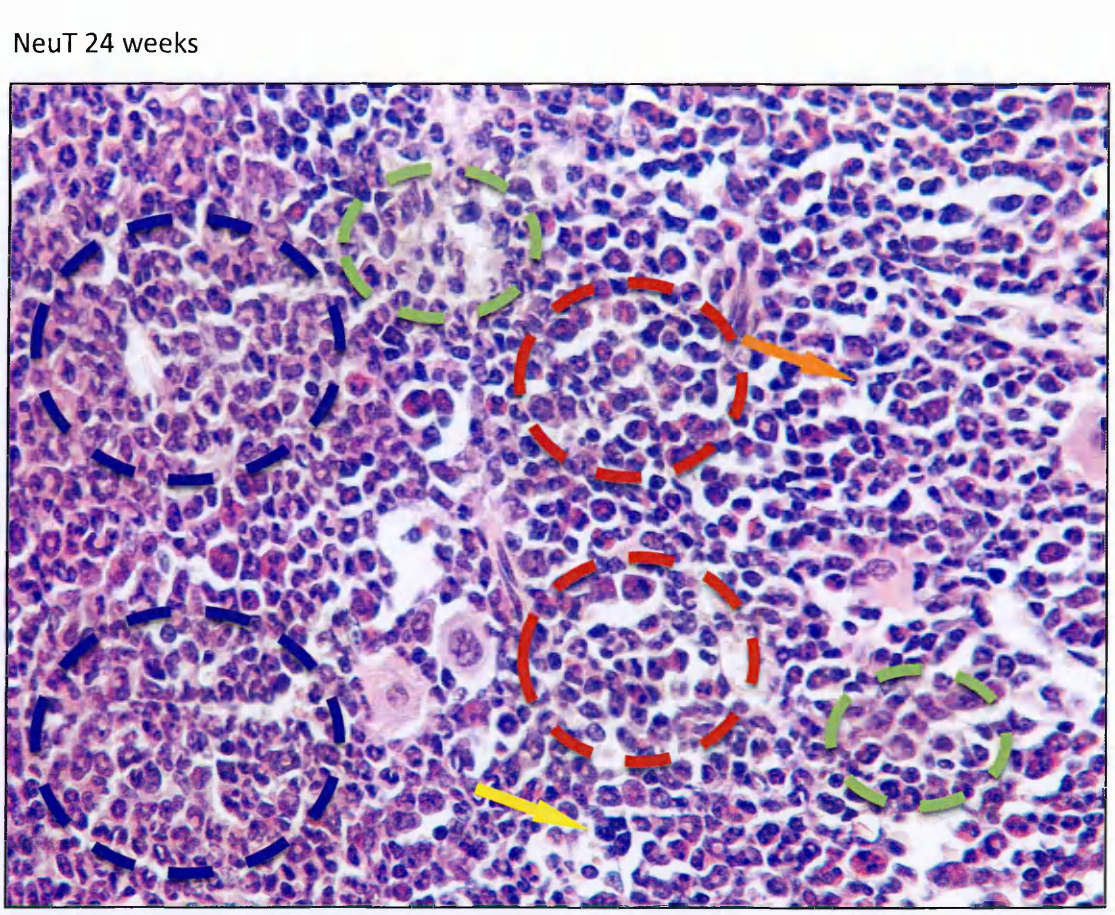


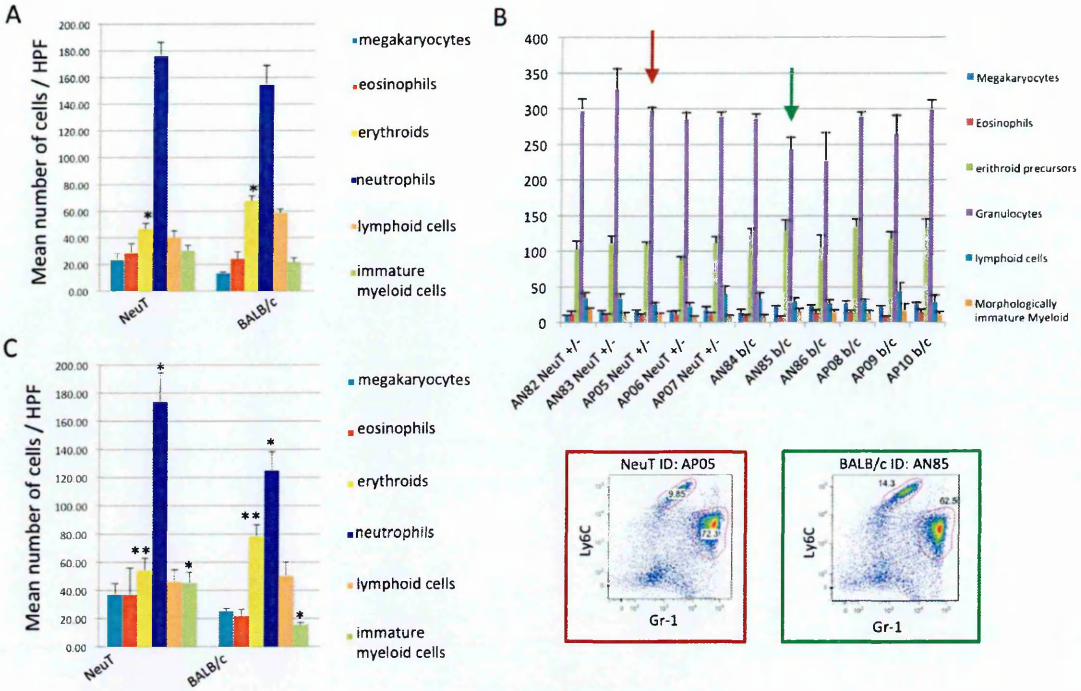
Figure 20 – Morphological analysis and quantification of immune populations of BM parenchyma at 24 weeks in NeuT mice.

A) Absolute number of cells per high power field (HPF) of mekagaryocytes, eosinophils, eryhtroids, neutrophils, lymphoid and immature myeloid cells in the BM of BALB/c and NeuT mice at 24 weeks of age. B) H&E staining in BM parenchyma of BALB/c and NeuT mice at 24 weeks of age and identification of eosinophils (red), eryhtroids (yellow), neutrophils (blue), lymphoid (orange) and immature myeloid cells (green).

Once myeloid expansion at late time points had been confirmed, hematopoietic cells count of BM sections have been performed in organs collected from mice with pre-cancerous lesions at 6 weeks of age. This analysis showed, in comparison to wt mice, a slight increase in morphologically immature myeloid precursors and a significant decrease in erythroid precursor clusters (Figure 21A) in line with the variations observed in mice with overt carcinoma. Moreover, an increase in neutrophil number also emerged from the analysis.

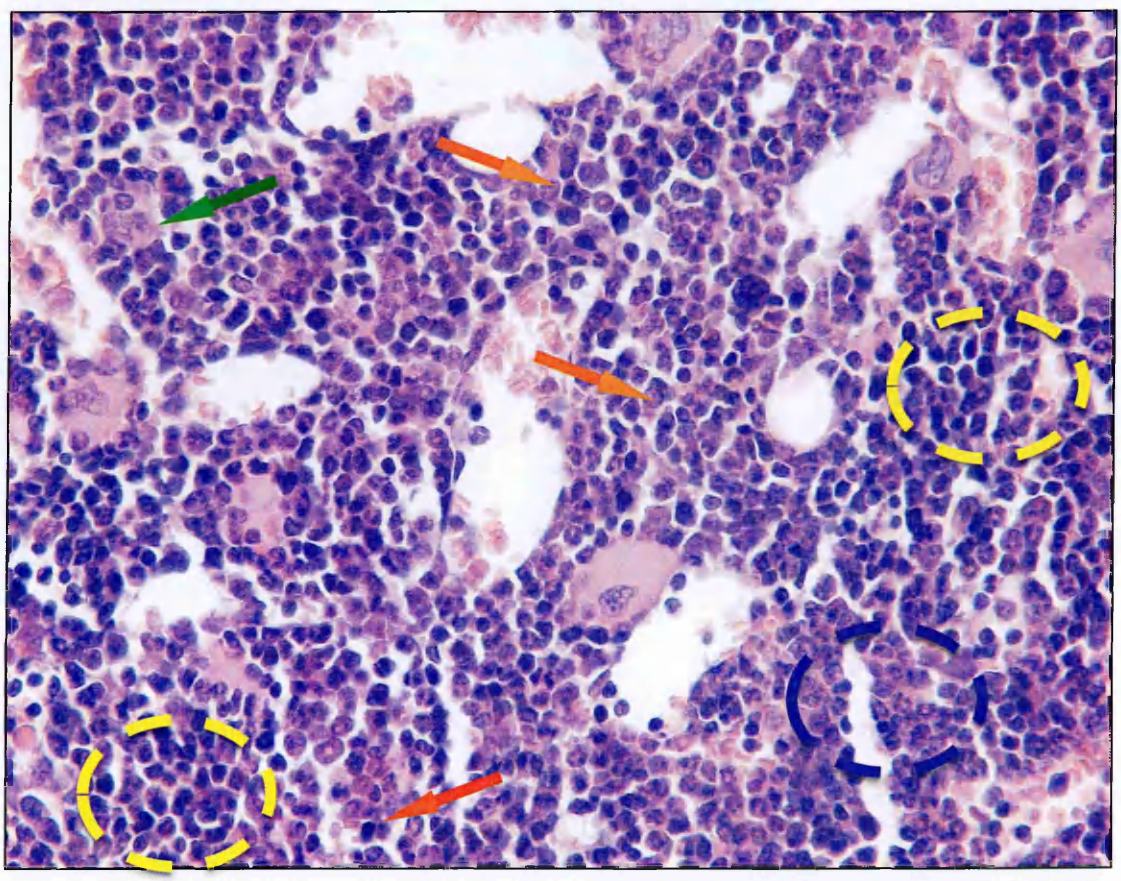
Notably, histopathology analysis finds concordance with FC sample by sample as shown by two representative samples in Figure 21B by the similar increment in granulocytic cells in NeuT compared to wt mice.

The difference in the BM became more evident along cancer progression at 12 weeks of age, as the increase in neutrophils and immature myeloid cells and the decrease in erythroid precursor were all proved to be statistically significant (Figure 21C-D).



D

BALB/c 12 weeks



NeuT 12 weeks

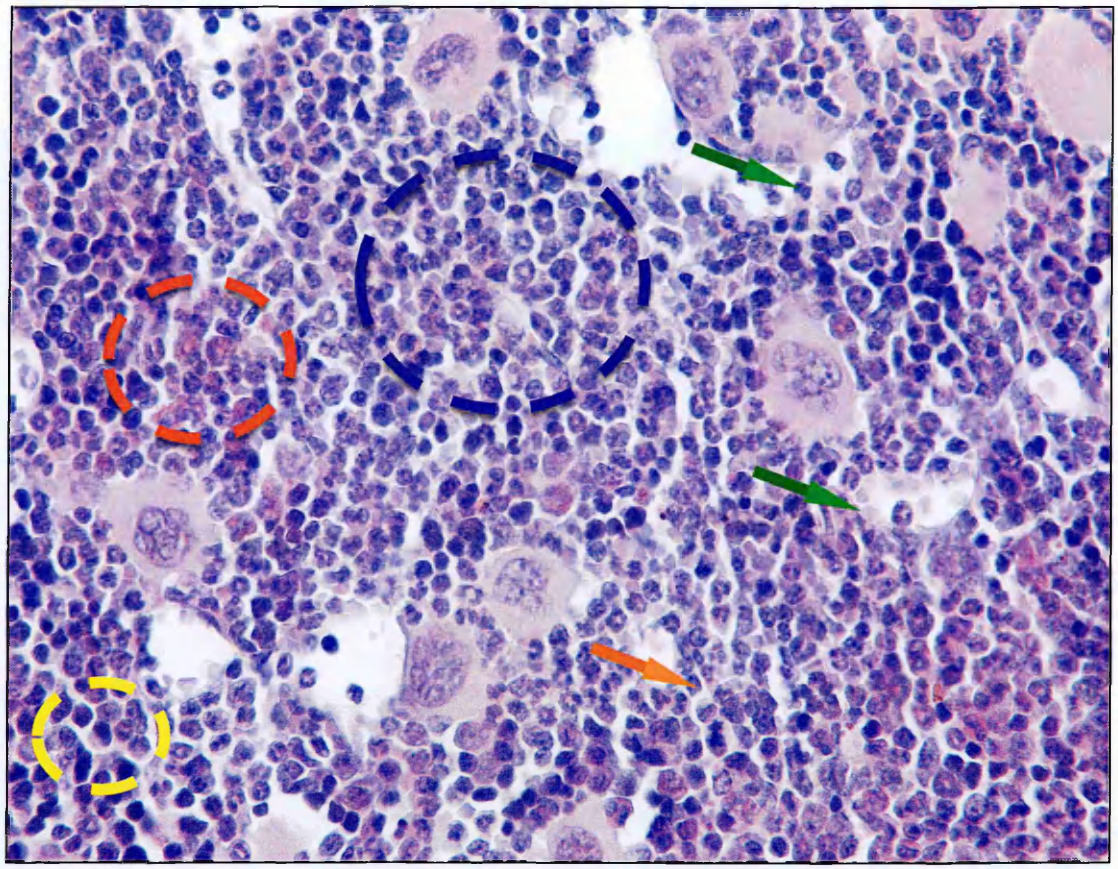


Figure 21 – Cell count and morphology of immune populations of BM parenchyma at 6 and 12 weeks in NeuT mice.

A) Mean number of cells per high power field (HPF) of mekagaryocytes, eosinophils, eryhtroids, neutrophils, lymphoid and immature myeloid cells in the BM of BALB/C and NeuT mice at 6weeks of age. B) Counts of cells at single mouse level in BALB/c and NeuT mice at 6 weeks of age and representative dot plots of FC analysis (lower panel) showing CD11b+ myeloid subset. C) Mean number of cells per high power field (HPF) mekagaryocytes, eosinophils, eryhtroids, neutrophils, lymphoid and immature myeloid cells in the BM of BALB/C and NeuT mice at 12 weeks of age. D) H&E staining in BM parenchyma of BALB/c and NeuT mice at 12 weeks of age and identification of eosinophils (red), eryhtroids (yellow), neutrophils (blue), lymphoid (orange) and immature myeloid cells (green).

In order to confirm the decrease in erythroid progenitors with a different approach, we focused on erythroid cells characterisation by FC analysis. The cell surface erythroid specific Ter119 antigen in expressed by terminally differentiating erythroblasts and, at intermediate levels, at the proerythroblast stage. Conversely, the transferrin receptor CD71, although not erythroid specific, is expressed at very high levels by early erythroid precursors and its level decreases with erythroid maturation. Using FC, we identified, as described in literature (Socolovsky, Nam et al. 2001), 4 distinct cell populations: proerythroblasts in the $Ter119^{med}CD71^{high}$ cell population (R1); basophilic erythroblasts as $Ter119^{high}CD71^{high}$ cells (R2); late basophilic and polychromatophilic erythroblasts as $Ter119^{high}CD71^{med}$ cells (R3); orthochromatophilic erythroblasts in the $Ter119^{high}CD71^{low}$ cell population (R4). All these subpopulation were gated on $B220^{-}CD11b^{-}$ cells (Figure 22A-B).

In agreement with IHC results, we observed a reduction in erythroid progenitors, in particular R1 and R2 subsets, in the BM of NeuT mice versus controls at 12 weeks of age. Spleen of the same mice showed a similar trend, although not statistically significant (Figure 22C).

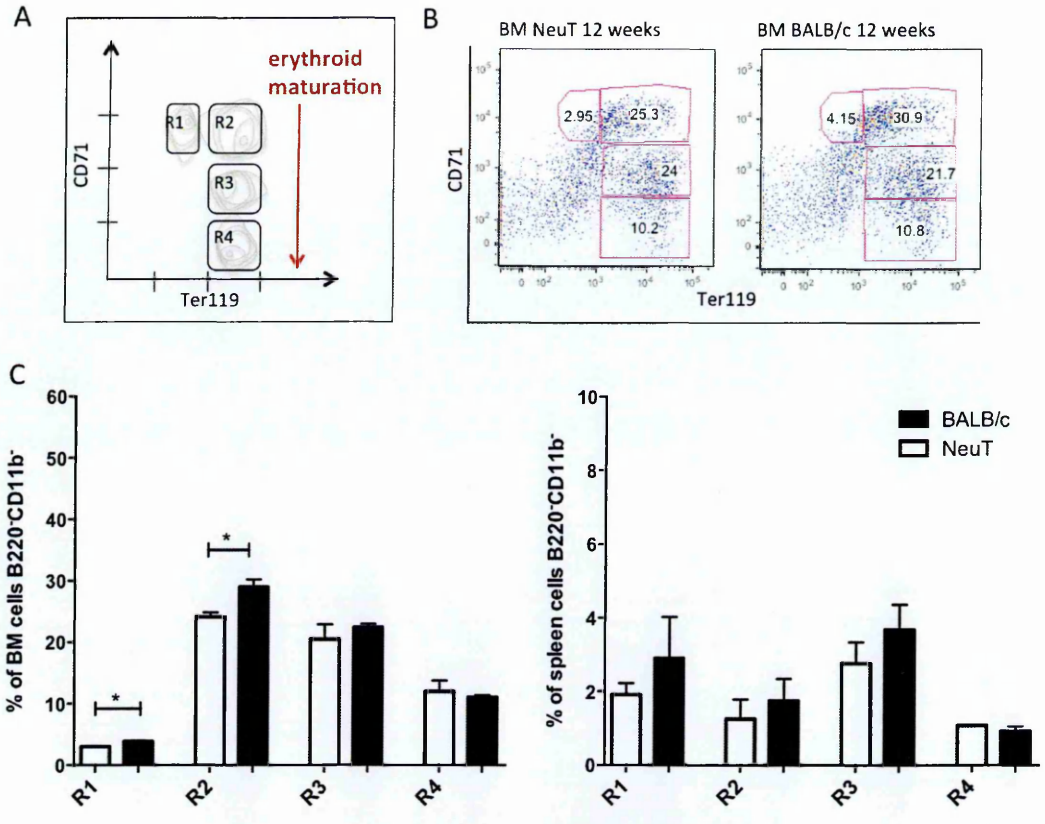
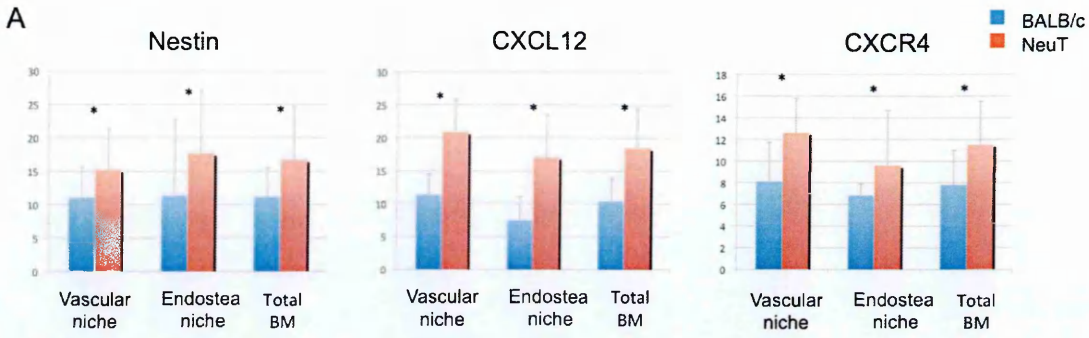


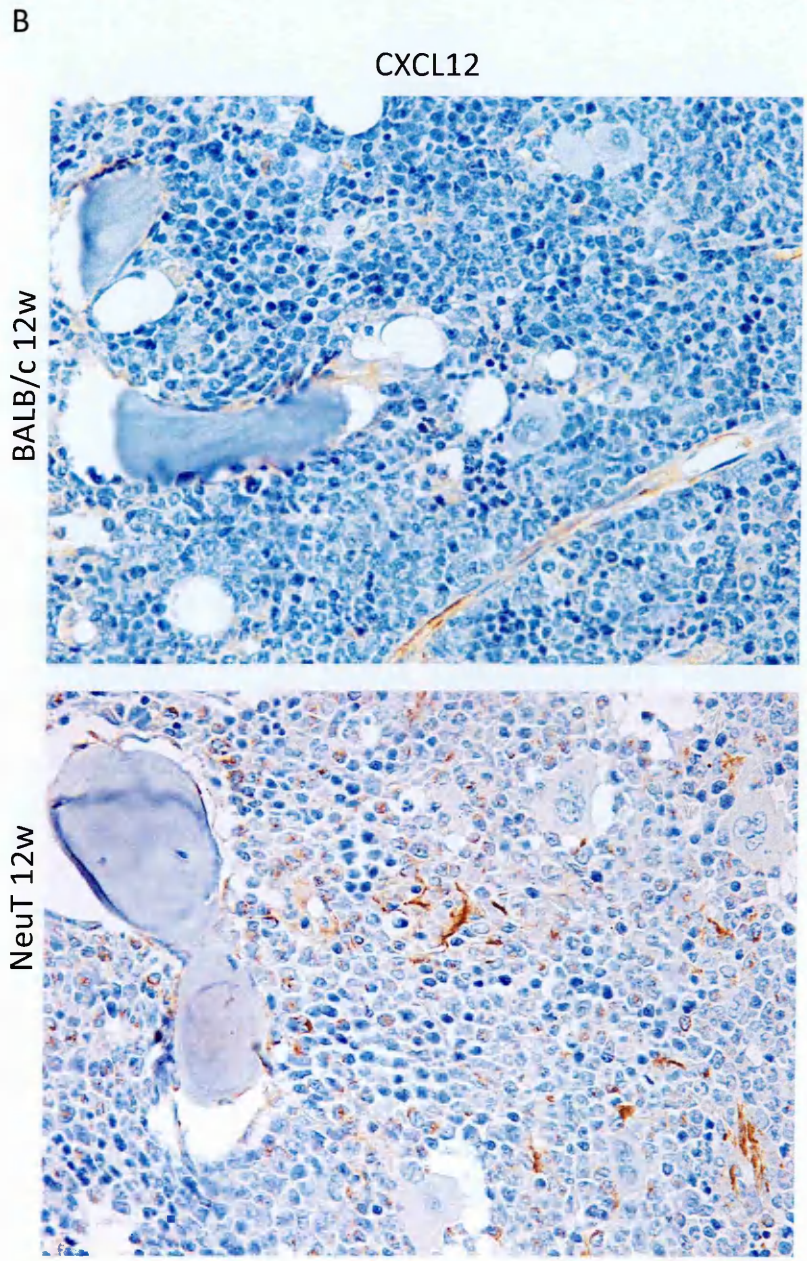
Figure 22 – Characterisation of erythroid subsets in NeuT mice at 12 weeks of age

A) Schematic view of FC plot for eryhtroid cells maturation: proerythroblasts (R1), basophilic erythroblasts (R2), late basophilic and polychromatophilic erythroblasts (R3) and orthochromatophilic erythroblasts (R4) gated on $B220^{-}CD11b^{+}$ cells. B) FC dot plots of erythroid cells in the BM of BALB/c and NeuT mice at 12 weeks of age. C) FC analysis of erythroid cells in BM (left panel) and spleen (right panel) in BALB/c and NeuT mice at 12 weeks of age. NeuT (n=3); BALB/c (n=3).

Through *in situ* immunolocalization analyses, we also observed that BM mesenchymal cells, which account for less than 5% of whole BM cellularity and that are hardly recovered by standard BM processing for FC analysis, underwent a significant modification in their density and localization already at 12 weeks. Indeed, Nestin+ BM mesenchymal cells expand in the BM osteoblastic (precursor-rich) and vascular (mature-cells-rich) niches in NeuT samples at 12 weeks and display significant up-regulation of the prototypical chemotactic stromal factor CXCL-12 at 12 weeks of age in BM of NeuT in comparison to wt mice.

Consistent with this finding, enrichment in CXCR4-expressing myeloid cells in the same BM areas of NeuT samples at 12 weeks was found (Figure 23A). We observed therefore a rearrangement in the CXLC12/CXCR4 axis: in particular in wt mice CXCR4 was linked to erythroid cells and the axis was preferentially expressed in the perivascular area, while in the NeuT parenchyma the axis co-localized with myeloid cells in the interstitial area of the BM (Figure 23B). Overall, the results highlighted that the early modifications occurring in the BM parenchyma at premalignant phases of cancer development mainly concern an innate immune cell subset, specifically granulocytic myeloid cells, that are enriched at the expense of other resident populations, being favoured in their repositioning within BM erythroid and lymphoid niches by the CXCL-12/CXCR-4 axis.


A
Nestin
CXCL12
CXCR4
BALB/c
NeuT
Vascular niche
Endostea niche
Total BM


B
CXCL12
BALB/c 12w
NeuT 12w

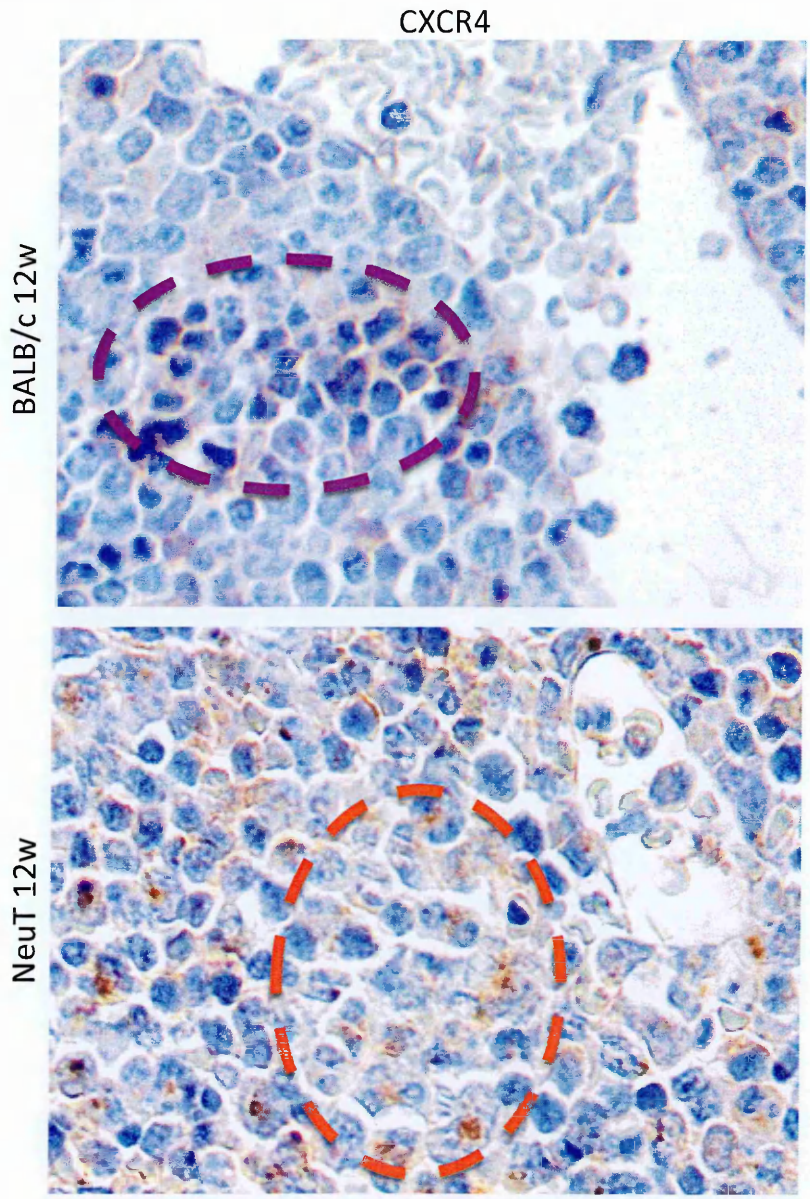


Figure 23 – Immunolocalization of nestin, CXCL12 and CXCR4 in BM of 12 week old NeuT mice

A) Quantitative immunolocalization analysis of nestin, CXCL12 and CXCR4 on BM vascular and endosteal niches of BALB/c and NeuT mice at 12 weeks of age. B) IHC analysis of CXCL12 and CXCR4 expressing elements in BM of BALB/c and NeuT mice at 12 weeks of age.

4.4 BM does not replace extramedullary function of the spleen in NeuT mice

With the idea of inducing in the BM a stress condition in order to accentuate the medullary haematopoiesis, spleen of NeuT mice has been surgically removed at 6 weeks of age and the BM has been analysed 10 weeks later.

The temporary window between the surgery and the analysis of the BM could allow the identification of tumour-progression related modifications with a higher magnitude in comparison to situation in which the spleen vicariates, at least in part, the BM-induced haematopoiesis. However, results from splenectomised mice did not revealed substantial changes in either differentiated or progenitor cells, suggesting that BM does not respond significantly in terms of increasing haematopoiesis after spleen surgical removal (Figure 24).

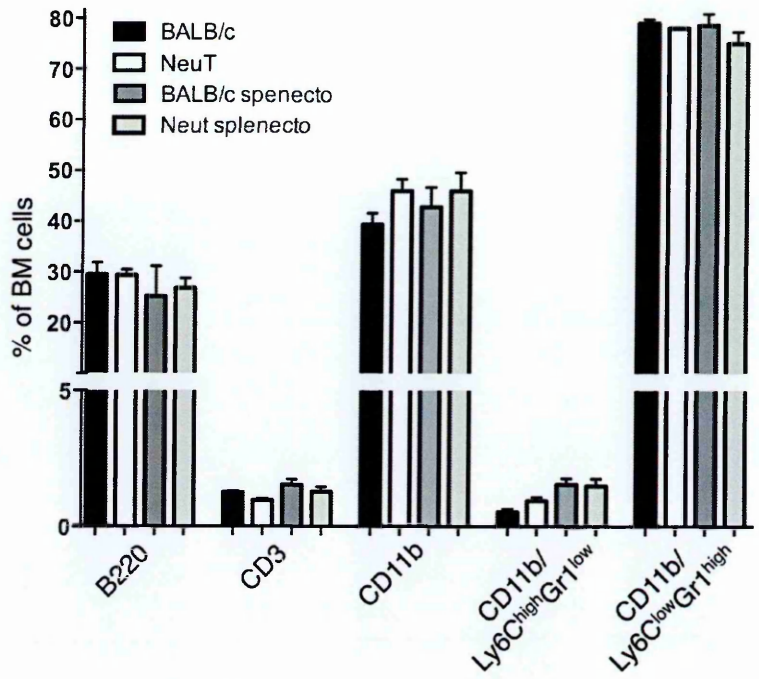


Figure 24 – Evaulation of leukocyte subsets in BM of splenectomised NeuT and wt mice

FC analysis of differentiated cells in BM for B220+ B cells, CD3+ T cells, myeloid CD11b+ cells and CD11bLyC/Gr1 subsets in BALB/c and NeuT mice subjected or not to splenectomy at 16 weeks of age. Each group n=3.

4.5 BM of tumour-bearing mice is characterized by immune and inflammation-related transcriptional programs

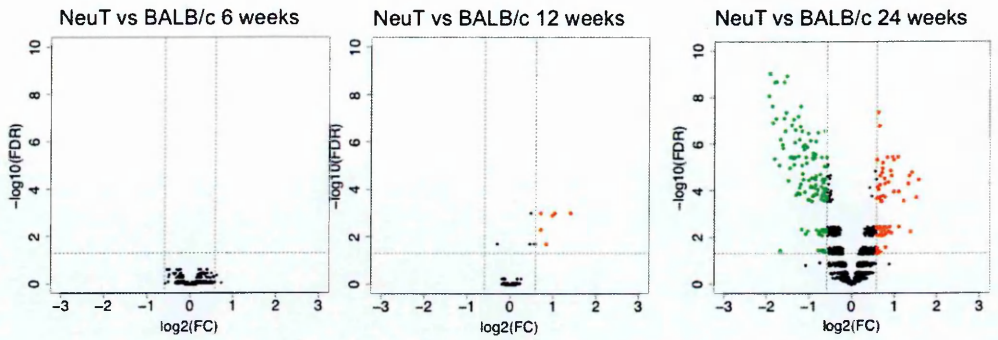
To gain further insight into early cancer-related BM changes at molecular level, we performed, in collaboration with the Functional Genomic and Bioinformatic Unit of our Institute, GEP on BM samples from the same mice analysed by FC and histopathology/IHC.

Firstly we performed a class comparison analysis in order to find which genes were differentially modulated (FDR < 0.05, Fold Change > 1.5) between NeuT and wt mice at each time point. No genes were differentially expressed at 6 weeks, instead, at 12 weeks of age 9 genes were up-regulated in the BM of NeuT mice in comparison to age-matched wt mice (Atf3, Trem1, Tnfaip3, Ccl4, Per1, Wdr92, Ncf2, Ddit3 and Clec4e). At 24 weeks of age, time point in which we expected pronounced differences between mice with overt tumours and healthy mice, the analysis showed 374 genes differentially expressed: 233 genes down-regulated and 141 genes up-regulated in NeuT samples versus controls (Figure 25A).

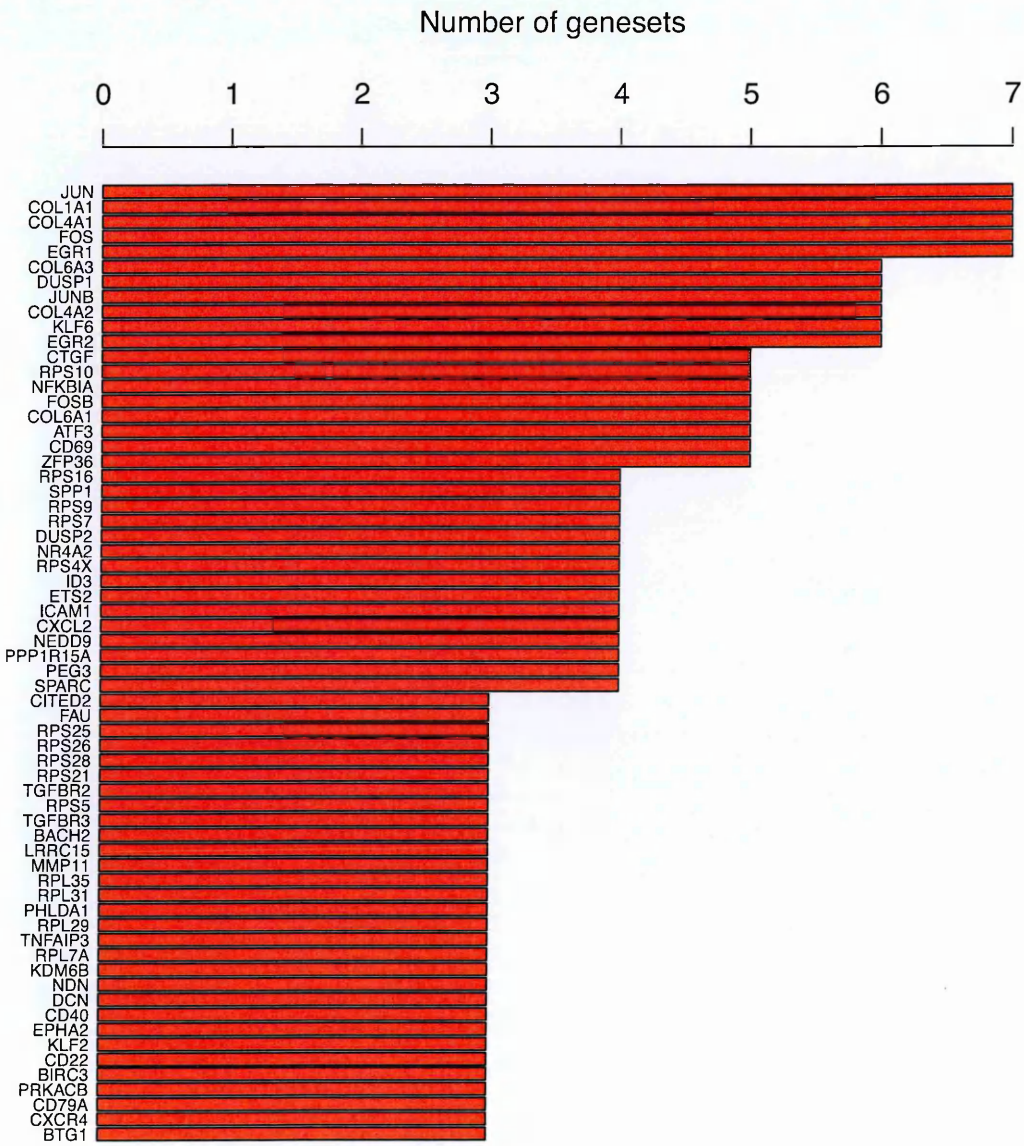
Using the computational method GSEA (gene set enrichment analysis), in which annotated gene sets are collected, is possible to evaluate if a gene set is correlated with the phenotypic class distinction. The gene set approach has the advantage of making easier the interpretation of the results by identifying pathways and processes and by detecting subtle enrichment signals. The primary result of this analysis is the enrichment score (ES), which reflects the degree to which a gene is over-represented at the top or bottom of a ranked list of genes, increasing the statistic when a gene is within the gene set. Data obtained with this approach confirmed genes such as Atf3 and Tnfaip3 positively enriched (in 5 and

3 gene sets respectively) in NeuT mice already at 6 weeks of age in comparison to wt mice (Figure 25B). Interestingly, genes concerning extracellular matrix and stroma remodeling were also positively enriched in tg mice at 6 weeks of age: Sparc, Spp1, Cxcr4 and collagen family members (Col1a1, Col4a1, Col6a1, Col6a3), supporting the *in situ* observation of stromal rearrangements preceding significant changes in haematopoiesis (Figure 25C).

A


NeuT vs BALB/c 6 weeks
NeuT vs BALB/c 12 weeks
NeuT vs BALB/c 24 weeks
−log10(FDR)
log2(FC)

B


Number of genesets
0 1 2 3 4 5 6 7
JUN
COL1A1
COL4A1
FOS
EGR1
COL6A3
DUSP1
JUNB
COL4A2
KLF6
EGR2
CTGF
RPS10
NFKBIA
FOSB
COL6A1
ATF3
CD69
ZFP36
RPS16
SPP1
RPS9
RPS7
DUSP2
NR4A2
RPS4X
ID3
ETS2
ICAM1
CXCL2
NEDD9
PPP1R15A
PEG3
SPARC
CITED2
FAU
RPS25
RPS26
RPS28
RPS21
TGFBR2
RPS5
TGFBR3
BACH2
LRRC15
MMP11
RPL35
RPL31
PHLDA1
RPL29
TNFAIP3
RPL7A
KDM6B
NDN
DCN
CD40
EPHA2
KLF2
CD22
BIRC3
PRKACB
CD79A
CXCR4
BTG1

C

Gene sets enriched at 6 weeks	COL1A1	COL4A1	COL6A1	SPP1	SPARC	COL6A3	FOSB	CXCR4
CROMER TUMORIGENESIS UP	X	X			X			X
WONG ADULT TISSUE STEM MODULE	X	X	X		X		X	
TURASHVILI BREAST LOBULAR CARCINOMA VS LOBULAR NORMAL DN	X	X				X		X
REACTOME SIGNALING BY PDGF	X	X	X	X		X		
REACTOME EXTRACELLULAR MATRIX ORGANIZATION	X	X	X			X		
CROONQUIST STROMAL STIMULATION UP			X		X	X	X	

Figure 25 – Differentially expressed genes in BM of NeuT and wt mice at different time points and enrichment analysis at 6 weeks of age.

A) Volcano plots of GEP analysis in the BM of NeuT mice in comparison to BALB/c mice at 6, 12 and 24 weeks of age with up-regulated (red) and down-regulated (green) differentially expressed genes. B) Leading edge analysis of GSEA in BM of NeuT mice at 6 weeks of age. C) Gene sets enriched in the BM of NeuT mice at 6 weeks of age and presence of ECM and stroma-related genes into each gene sets.

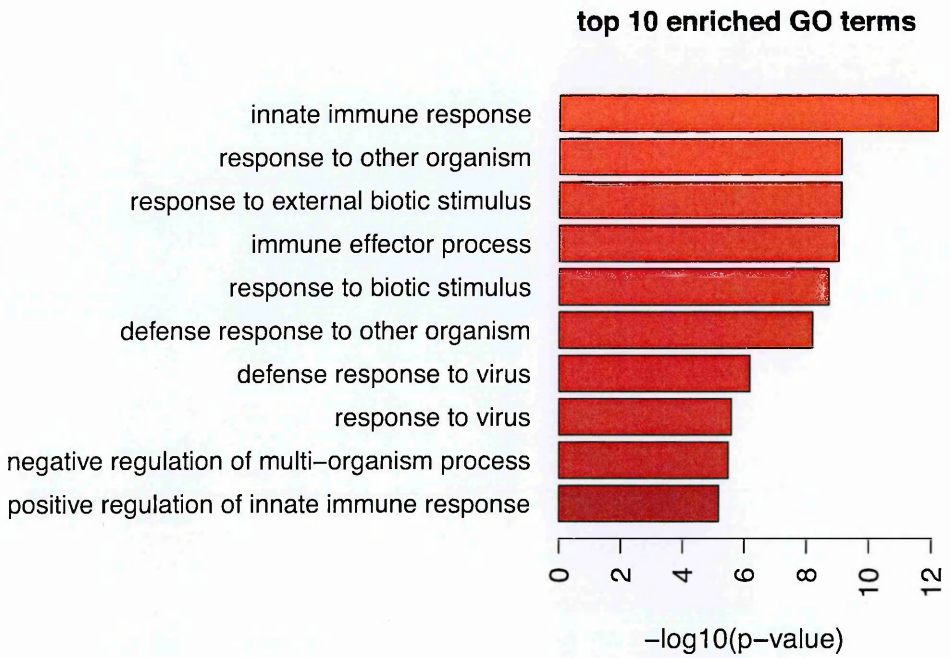
As expected from class comparison analysis, the major contribution in terms of genes differentially expressed is provided by late time point samples and the up- and down-regulated genes were used to build a "late BM gene signature" in order to be applied to earlier time points and to validation cohorts. Gene Ontology analysis was applied to the up- and down-regulated gene sets of the signature and the top 10 biological process domains (FDR < 0.05) were selected as representative of the modulated BM transcriptional programs. Among the up-regulated genes in NeuT mice we found enrichment for innate immune response and inflammatory response programs (Figure 26A). Down-regulated genes, on the other hand, included adaptive immune response and B cell receptor signalling pathway, in agreement with reduction of B cells compartment in the BM of the same mice observed by FC (Figure 26B).

Using the results which emerged from the class comparison analysis at 24 weeks of age, we decided to take advantage of GSEA approach running the late BM signature, considering the down-regulated genes and the up-regulated genes as independent gene sets, against 6 and 12 weeks datasets. Interestingly, an inverse correlation was found between genes up and down-regulated at 6 weeks indicating that BM samples of mice with very early pre-malignant lesions did not share the inflammation-related profile proper of overt malignancy (Figure 26C). Importantly, at 12 weeks of age, the same type of analysis showed a direct correlation with late gene signatures indicating that an inflammatory switch in BM myelopoiesis and a decreased in B cell signalling and immune response-related pathways occurred at the stage of dysplasia/*in situ* cancer (Figure 26D). Together the results suggest that the BM attempts to correct stroma modification, which begins very early at

transformation stages, and the pronounced differences characterizing the stroma of tumour-bearing mice already mark the BM collected at 12 weeks of age.

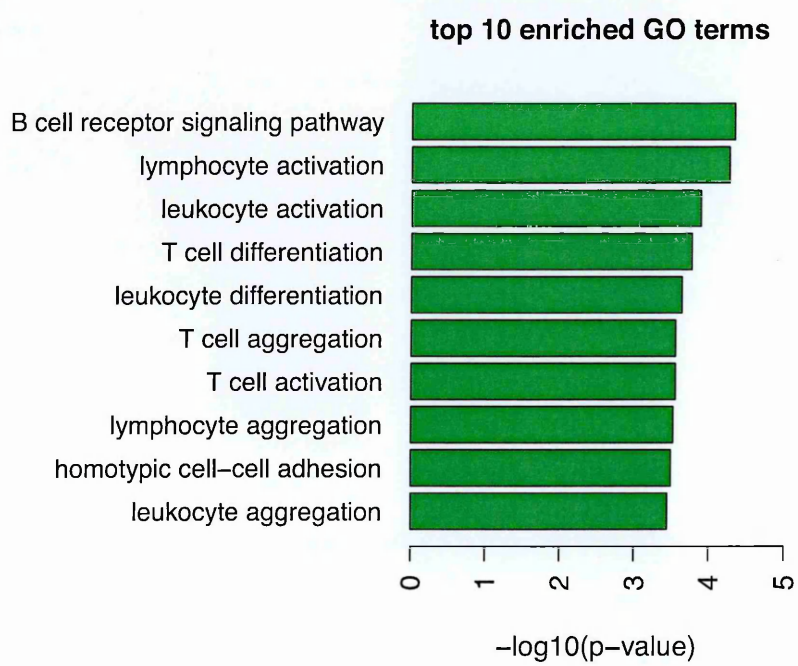
A

Inflammatory and innate immune response processes



B

Adaptive immune response, B cell receptor signaling and lymphocyte differentiation/activation



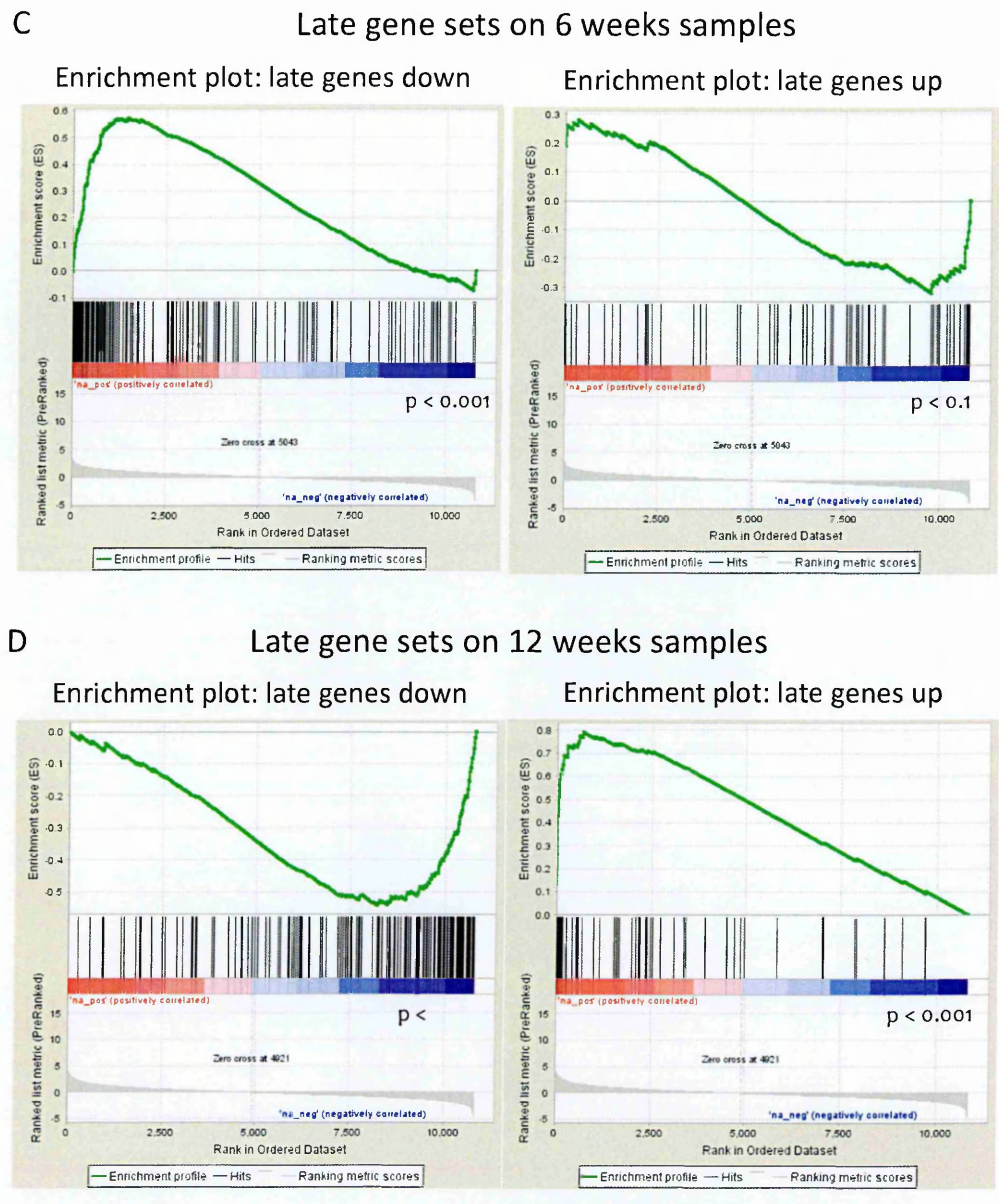


Figure 26 – Late BM gene signature-related processes and their enrichment at early time points

A-B) Barplots showing the top 10 significantly enriched Gene Ontology terms (biological process domain) in the list of up- (A) and down-regulated (B) genes of the late BM late signature.

C-D) Enrichment score for the late BM gene signature in the GEP data of BM of NeuT mice at 6 (C) and 12 (D) weeks of age.

Furthermore, taking advantage of Cibersort, a recent analytic tool developed by Newman and colleagues, which, using gene expression data, provides an estimation of the different immune cell types in a mixed human cell population, we could estimate the participation of the different immune cell subsets in the BM transcriptional programs of NeuT and BALB/c mice at 24 weeks of age. Being aware that the Cibersort signature matrix (LM22) is human-based and built considering 22 different immune cell subsets with their specific expressed genes, a degree of conservancy of specific immune cells signatures is observed, making this approach at least in part reliable on mouse settings. Indeed Cibersort analysis indicated a contraction of B lymphocytes and an accumulation of neutrophils in NeuT in comparison to wt mice (Figure 27), further confirming our histopathological analyses revealing a myeloid cell-skewed haematopoiesis and FC data supporting B cell pool contraction.

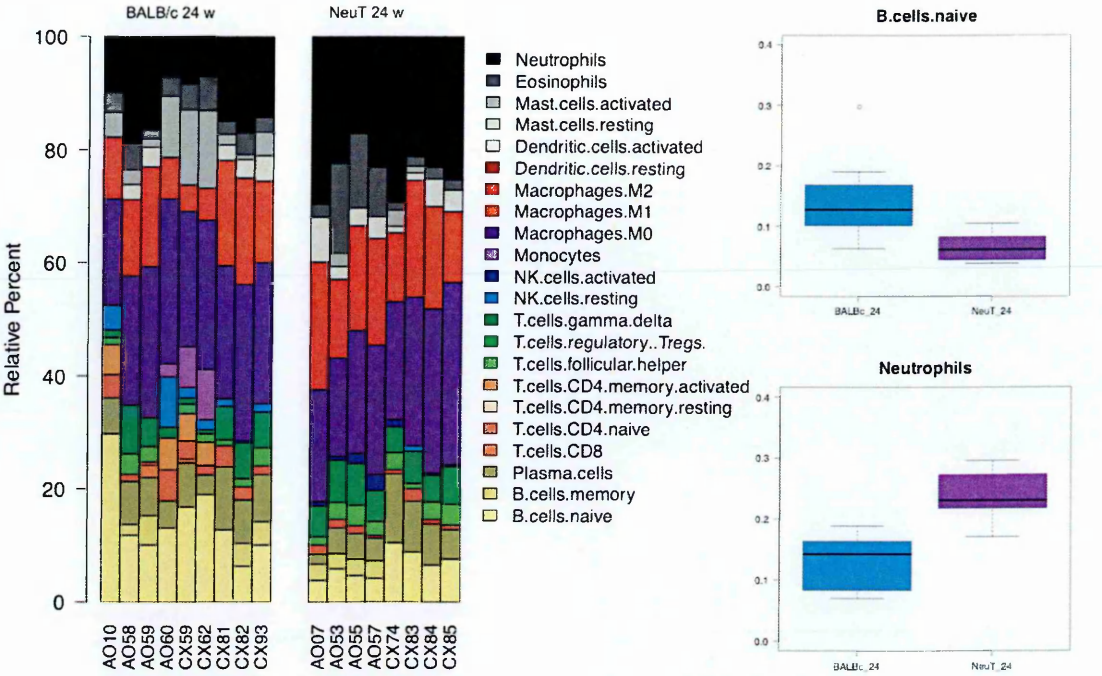


Figure 27 – Application of Cibersort tool on BM of NeuT and wt mice at 24 weeks of age

Immune cell composition of BM of BALB/c and NeuT mice at 24 weeks of age (left panel) and naive B cells and neutrophils-related box plots (right panels). Cibersort tool provides an estimation of different immune cell subsets using gene expression data.

To validate the data obtained from the first GEP profile (discovery set) we collected a new cohort (validation set) of NeuT mice together with control siblings, at the same time points analysed so far. In order to technically validate the results, some BM samples at 24 weeks analysed in the previous GEP have been hybridized together with the new cohort of mice at the same age. Sixty-nine differentially expressed genes resulted to be in common between discovery and validation set at late time point (Figure 28A) and GSEA, using the up- and down-regulated genes of the discovery set against the late BM samples of the validation set, showed a perfect overlap between the two cohorts underlining the same modulation in terms of enriched genes (Figure 28B). In the second GEP, several genes were found differentially expressed already at 6 weeks of age: in particular, 114 genes were down-regulated and 6 genes up-regulated in NeuT mice in comparison to wt mice (FDR < 0.05, Fold Change > 1.5). On the other hand, unexpectedly, at 12 weeks we did not observe any modulation in the GEP between tg and wt mice. At late time point, as expected, we found several modulated genes in NeuT versus BALB/c mice. In particular 54 genes were up-regulated and 55 genes were down-regulated (Figure28C).

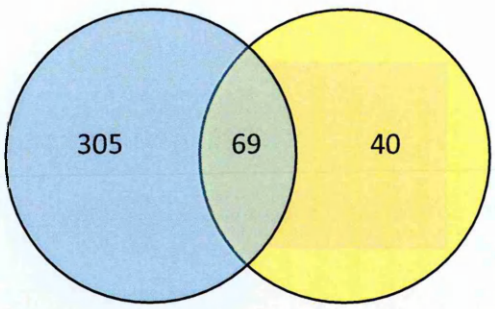
To assess whether the modulations observed in the NeuT mice were specific of that model or could have a broader significance, GEP has also been performed on different breast cancer models, PyMT and huHer2Δ16. Results concerning huHer2Δ16 BM samples highlighted two genes differentially expressed between transgenic and wt samples already at 9 weeks of age [Defb15: Fold Change = 1.41, Col6a3: Fold Change = -2.2], considering as relevant genes with a p value <

0.001. At 12 weeks of age, 62 genes were differentially expressed whereas at late time point (24 weeks of age) 50 genes were modulated (Figure 28D).

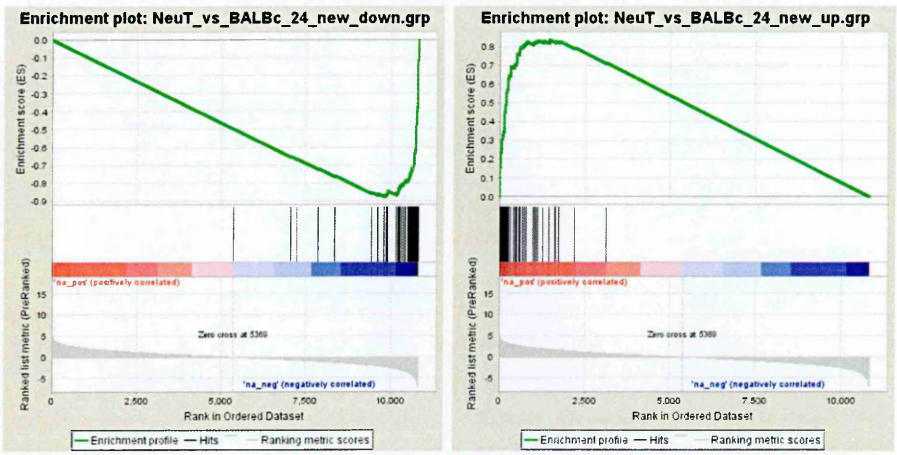
The same analysis performed on BM of PyMT mice showed 7 genes differentially expressed at 6 weeks of age. At 12 weeks of age 3 genes were modulated (Cpeb1: Fold Change = 1.2, Nudt7: Fold Change = -1.2, Atg3: Fold Change = -1.7) while at 24 weeks of age 52 genes were differentially expressed between the two classes (Figure28E).

These results suggest that, at early time points during tumourigenesis, BM modifications, in terms of gene expression, are specific for the different transgenic models.

A


Late discovery set
Late validation set
305
69
40

B


Enrichment plot: NeuT_vs_BALBc_24_new_down.grp
Enrichment score (ES)
Ranked list metric (PreRanked)
'na_pos' (positively correlated)
Zero cross at 5369
'na_neg' (negatively correlated)
Rank in Ordered Dataset
Enrichment profile
Hits
Ranking metric scores
Enrichment plot: NeuT_vs_BALBc_24_new_up.grp
Enrichment score (ES)
Ranked list metric (PreRanked)
'na_pos' (positively correlated)
Zero cross at 5369
'na_neg' (negatively correlated)
Rank in Ordered Dataset
Enrichment profile
Hits
Ranking metric scores

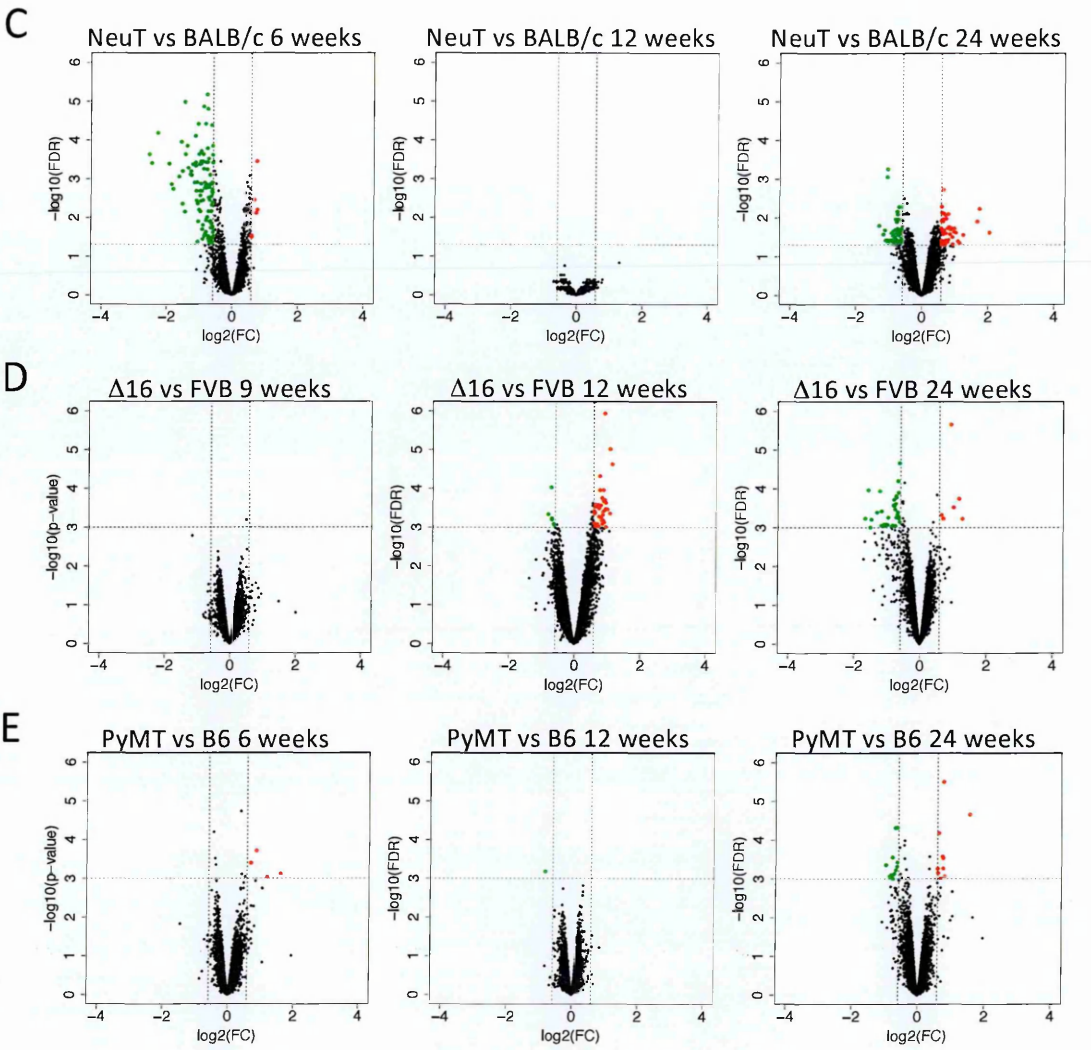


Figure 28 – Gene expression profile on validation set and in NeuT, PyMT and huHer2Δ16 mice and wt mice

A) Venn diagram of differentially expressed genes in NeuT mice at 24 weeks of age between discovery and validation set. B) GSEA of the late BM gene signature in NeuT validation set at 24 weeks of age. C) Volcano plots of GEP analysis of the validation set in BM of NeuT mice in comparison to BALB/c mice at 6, 12 and 24 weeks of age. D) Volcano plots of GEP analysis in BM of huHer2Δ16 (Δ16) mice in comparison to FVB mice at 9, 12 and 24 weeks of age. E) Volcano plots of GEP analysis in BM of PyMT mice in comparison to B6 mice at 6, 12 and 24 weeks of age. Differentially expressed genes are represented in the Volcano plots as up-regulated (red) and down-regulated (green) dots.

4.6 BM late signature discriminate tumour-bearing mice from healthy mice in NeuT, PyMT and huHer2Δ16 mice

As previously described genes differentially expressed between NeuT and BALB/c mice at 24 weeks of age of the discovery set (first cohort) were used to build a late BM gene signature. According to the signature, the samples of validation cohort were clustered and an optimal separation between NeuT and BALB/c groups was achieved (Figure 29A), which demonstrated that the discrimination between tg and wt mice is achievable according to BM gene expression profiles. GSEA, using late differentially expressed genes obtained in NeuT model, was also applied to late PyMT (Figure 29B) and huHer2Δ16 (Figure 29C) samples revealing that common BM gene expression pathways are enriched (positively or negatively) in all these three models upon overt cancer development, despite different driving oncogenes and genetic backgrounds.

In addition the late BM gene signatures have been compared with a new cohort of mice with first signs of palpable tumours and the results highlighted a good correlation with the up- and down-regulated genes (Figure 29D). This confirmed that late BM signature is able to distinguish tg from wt mice with both advanced cancer and at first signs of palpable tumour.

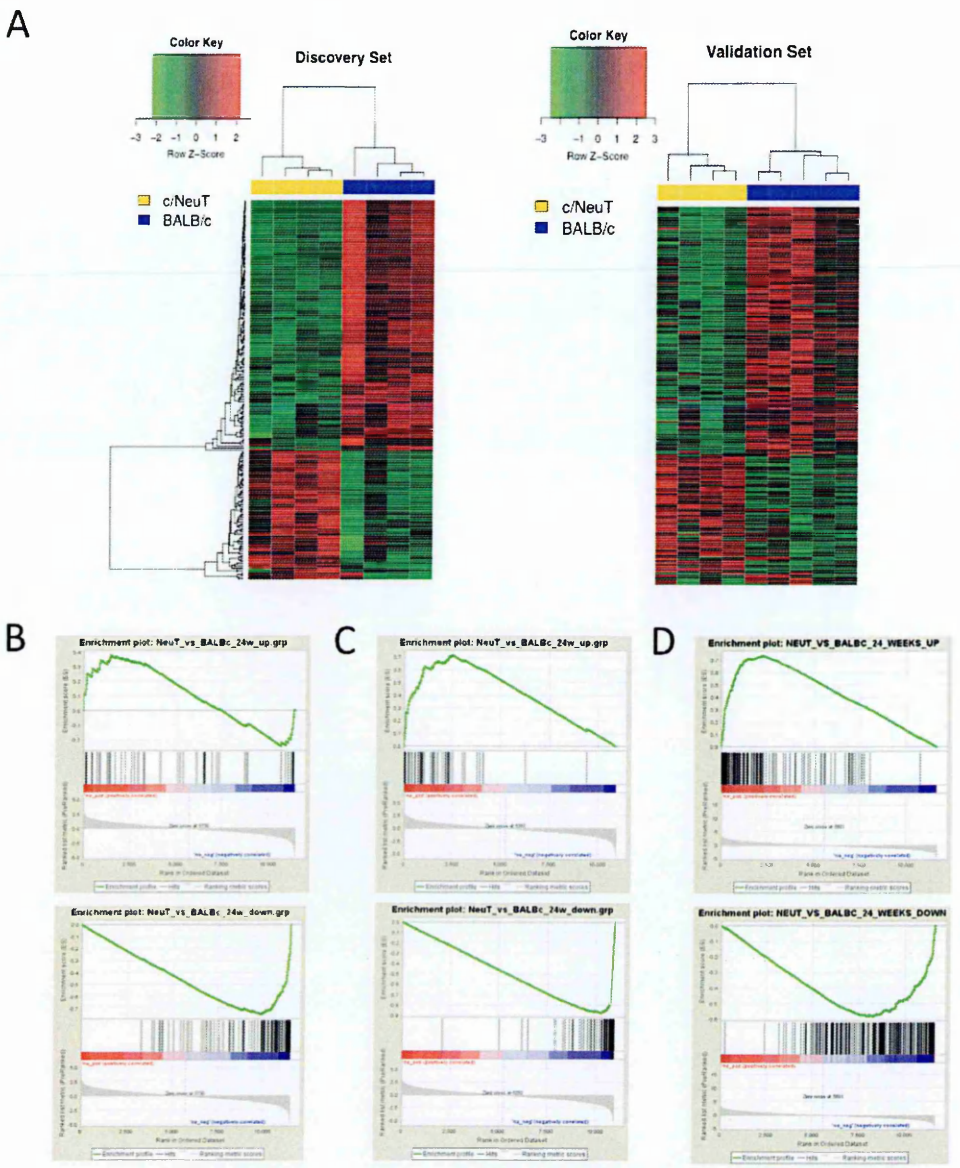


Figure 29 – BM late gene signature discriminates tumour bearing mice from wt mice

A) Heatmaps showing the expression pattern of the late BM gene signature on BM of NeuT mice at 24 weeks of age of the discovery set (left panel) and of the validation set (right panel).
GSEA of the late BM gene signature in PyMT mice at 24 weeks of age (B), in huHER2Δ16 mice at 24 weeks of age and in NeuT mice with first sign of palpable tumours (C).

4.7 Re-classification of early samples on the bases of mammary glands severity using 4 histopathological categories and an adjusted score

The data obtained from the two cohorts by GEP analyses of the two cohorts (discovery and validation set) were discrepant at early time points (6 and 12 weeks of age) in terms of differentially expressed genes between NeuT mice and non-transgenic siblings. The reason of such discrepancy could stem from the individual variability in the onset and extension of nascent lesions within the mammary glands of the mice. Indeed, although the first and major driver of tumour development in all NeuT mice is the activated rat Neu oncogene, additional mutations/alterations are required in the transforming cell to give rise to a full-blown cancer and these events could differ in the quality and timing from mouse to mouse. This could lead to some variability within the model in terms of both severity and extension of the disease at specific time points. To test our hypothesis, a throughout histopathological analysis was performed by the group of Prof. Tripodo on mammary gland samples from NeuT and wt mice at 6 and 12 weeks of age and the severity of mammary gland tissue changes was graded according to an ad-hoc histopathological score based on the assessment of the following variables: ductulo-lobular epithelial hyperplasia, epithelial dysplasia, carcinomatous transformation, peri-ductal inflammatory infiltration, distant stroma inflammatory infiltration, peri-ductal stromal remodeling. For each of the variables analysed, both the degree and the extension of changes were scored.

Morphological and histological analyses on mammary glands of NeuT and BALB/c samples at early time points showed different grade and extension of such

parenchyma modifications. NeuT mice showed a certain degree of variability in the severity of pre-malignant and/or early cancerous lesions with some cases showing signs of accelerated progression, i.e. NeuT samples at 6 weeks showing severe dysplastic changes and NeuT samples at 12 weeks of age with foci of microinvasive carcinoma, while other NeuT cases characterized by a rather indolent rate (Figure 30).

normal	mild/moderate dysplasia	severe dysplasia	early carcinoma
AM32 wt 12 w	AM30 wt 12 weeks	AO34 NeuT 12 w	AM27 NeuT 12 w
AO35 wt 12 w	AM31 NeuT 12 w	AN81 NeuT 6 w	AM28 NeuT 12 w
AO36 wt 12 w	AM 09 wt 6 w	CX44 NeuT 6 w	AM29 NeuT 12 w
AO37 wt 12 w	AM 15 wt 6 w	CX58 NeuT 6 w	AO31 NeuT 12 w
AO38 wt 12 w	AM 16 wt 6 w	DM60 NeuT 12 w	AO32 NeuT 12 w
AM74 wt 12 w	AM 17 wt 6 w	DM61 NeuT 12 w	AM71 NeuT 12 w
AM75 wt 2 w	AN84 wt 6 w		AM72 NeuT 12 w
AM76 wt 6 w	AN85 wt 6 w		AM73 NeuT 12 w
AM 07 wt 6 w	AM 12 NeuT 6 w		AN82 NeuT 6 w
AM 11 wt 6 w	AM 14 NeuT 6 w		AM 06 NeuT 6 w
AN86 wt 6 w	CX42 NeuT 6 w		AM 08 NeuT 6 w
CX46 wt 6 w	CX43 NeuT 6 w		AM 10 NeuT 6 w
CX47 wt 6 w	CX45 NeuT 6 w		CX36 NeuT 12 w
CX55 wt 6 w	CX56 NeuT 6 w		CX40 NeuT 12 w
CX37 wt 12 w	CX54 wt 5 w		DM06 NeuT 12 w
CX38 wt 12 w	CX57 wt 6 w		DM07 NeuT 12 w
CX39 wt 12 w	CX32 NeuT 12 w		DM09 NeuT 12 w
CX41wt 12 w	CX33 NeuT 12 w		DM33 NeuT 12 w
DM05 wt 12 w	CX34 NeuT 12 w		DM34 NeuT 12 w
DM63 wt 12 w	CX35 NeuT 12 w		DM59 NeuT 12 w
DM64 wt 12 w	DM04 wt 12 w		DM62 NeuT 12 w
DM65 wt 12 w	DM32 wt 12 w		DN47 NeuT 12 w
DM67 wt12 w	DM66 wt 12 w		DN53 NeuT 12 w
DN50 wt 12 w	DN49 wt 12 w		AM13 NeuT 6 w
DN52 wt 12 w	DM08 NeuT 12 w		AO33 NeuT 12 w
DN54 wt 12 w			

Figure 30 – Reclassification of BM samples based on mammary gland disease severity

Re-classification of BM early samples (6 and 12 weeks) of BALB/c (wt) and NeuT mice on the bases of the disease severity (normal, mild/moderate dysplasia, severe dysplasia and early carcinoma). Each mouse is described by the identification number, strain and age.

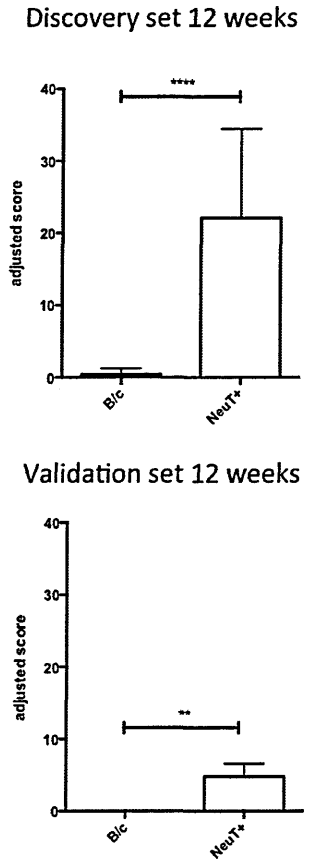
Based on the above histopathological analyses, we have firstly developed an "adjusted score" to re-modulate the two classes taking into account the contribution of the grade/extension of dysplasia and of the grade/extension of carcinoma, giving a higher contribution to samples with the presence of cancer, and not only dysplasia.

Following this consideration, we settled the formula for the adjusted score as = (dysplasia grade + dysplasia extension) * [1+(carcinoma grade * carcinoma extension)]. Using this adjusted score, the severity and extension of the lesions in NeuT samples of 12 weeks in comparison to non-transgenic siblings in the two cohorts are significantly different, being much higher in the first set of samples and therefore possibly explaining the lack of gene modulation we have observed at 12 weeks of age in the second cohort (Figure 31A).

Since we observed a considerable degree of individual variability in the onset and extension of nascent lesions within the mammary glands of animals, we have re-classified all BM samples from early time points on the basis of the degree of epithelial dysplasia, independently from age or genotype of mice. We sorted early samples into 4 different histopathological categories with increasing severity: normal (no dysplasia), mild or moderate dysplasia (dysplasia score: 1 or 2 respectively), severe dysplasia (dysplasia score: 3) and early carcinoma (carcinoma in situ/microinvasive carcinoma). This allowed us to compare, with a higher resolving power, these classes in order to identify which tissue transition had the major role in terms of differentially expressed transcripts in the BM. Class comparison analysis between early carcinoma and normal tissue showed that 18 genes are differentially modulated, 12 up-regulated and 6 down-regulated.

Notably, some of them, such as Atf3 and Tnfaip3 had been already identified as differentially expressed in NeuT and BALB/c mice according to the discovery data set at 12 weeks of age (Figure 31B).

A



B

Gene symbol	Fold Change
Atf3	1.61
Rgs1	1.49
Wdr92	1.36
Tnfaip3	1.35
Klk1	-1.35
Klk1b27	-1.33
Cd209f	1.32
Vsig4	1.31
Klk1b26	-1.3
Ppp1r15a	1.29
Hist1h2bc	1.28
Cldn10	-1.27
Fam46c	1.27
Cd8b1	-1.26
Egr2	1.24
Hist1h3d	-1.24
Arl4a	1.23
Pmaip1	1.2

Figure 31 – Application of the adjusted score on early mammary glands and differentially expressed genes in the BM of re-classified samples

A) Comparison between BALB/c and NeuT samples of the discovery and validation set based on the assessment of adjusted score. B) Ranked genes differentially expressed and relative Fold Change in the comparison between mice with early carcinoma and mice with normal tissue.

4.8 BM late signature is able to distinguish mice with normal/mild dysplasia in their mammary glands from those with severe dysplasia or early carcinomas

Unsupervised clustering analysis, using the late BM gene signature, considering genes with Fold Change > 2, on early samples (only 6 and 12 week time points), showed two clusters, clearly distinguishable in the dendrogram (Figure 32). These two clusters very efficiently separate early BM samples of mice that have normal/mild dysplasia in their mammary glands from those with severe dysplasia/early carcinomas. Notably, even if no differences in transcripts have been observed with class comparison analysis between NeuT and BALB/c mice at 6 weeks, the late BM gene signature is able to clearly distinguish NeuT from BALB/c samples at this very early time point.

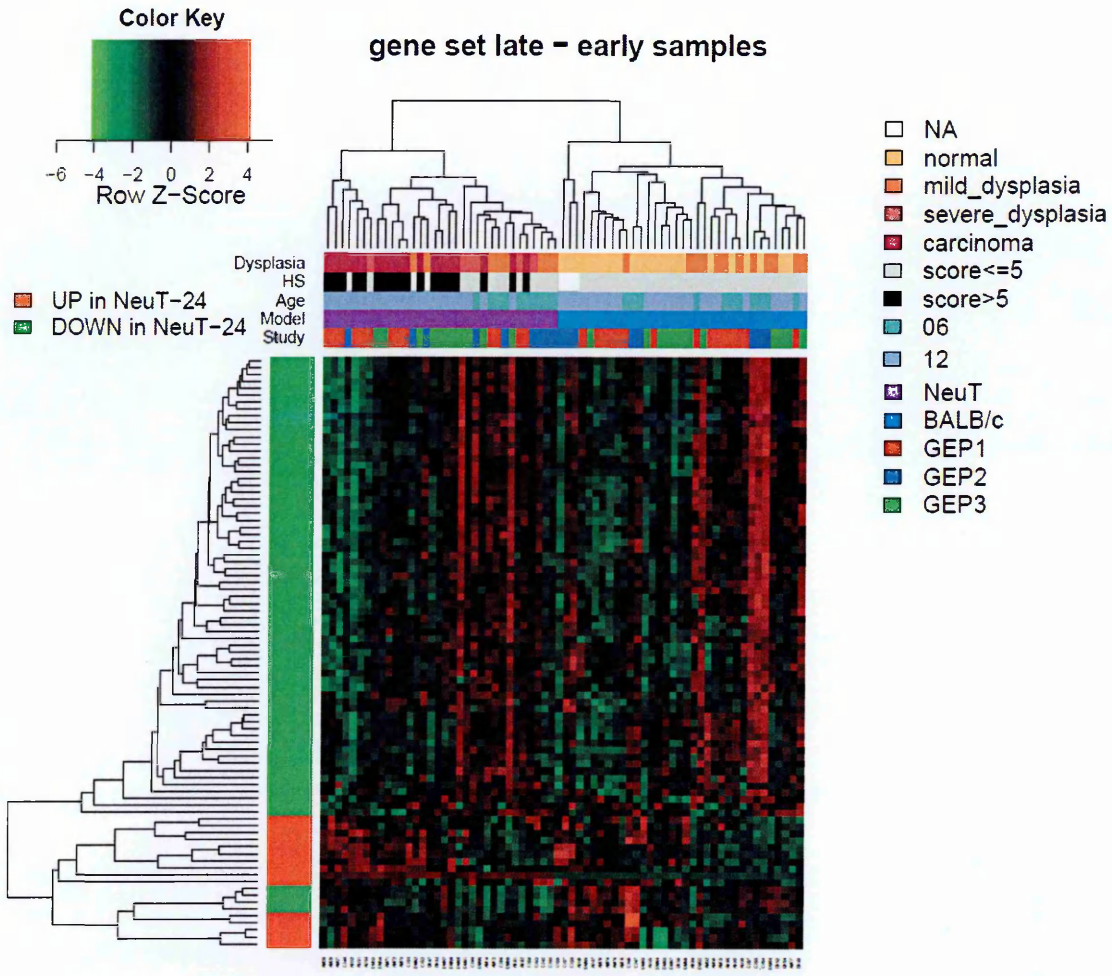


Figure 32 – Unsupervised clustering analysis using BM late signature on early samples

BM late gene signature (inclusion of genes differentially expressed with a lfold changel ≥2) on the early samples is able to distinguish mice with normal/mold dysplasia in their mammary glands from those with severe dysplasia or early carcinomas.

4.9 BM and plasma of NeuT mice display up-regulated microRNAs

To investigate the signals potentially responsible for the induction of early BM changes, microRNA analysis has been performed firstly on the same BM samples of the first GEP and then on plasma samples from the same mice. A comparison between BM of NeuT and wt mice at each time point revealed differences only at 24 weeks of age, whereas at early stages of progression the analysis did not show any statistically significant differences between the groups (Figure 33A). At the late time point (24 weeks of age), 21 microRNAs were differentially modulated according to a false discovery rate (FDR) < 0.05 and to a Fold Change > 1.15. Among these microRNAs 14 were up-regulated and 9 were down-regulated in NeuT mice in comparison to control mice. The most up-regulated microRNAs are mmu-miR-21a-5p and mmu-miR-146b-5p (Fold Change = 1.66 and 1.31 respectively). Some of the differentially expressed microRNAs have been already described to have a role in tumour settings and in inflammation processes.

The plasma from the same NeuT and wt mice of the first cohort, has been profiled for circulating microRNAs. In order to avoid the possibility that some microRNAs, being released after lysis of erythrocytes, could potentially impact the result, we settled up an experimental study to takes into account the issue of haemolysed samples. To this aim, we included in the case series a group of NeuT samples and related controls in which we experimentally forced or not the haemolysis in order to evaluate the microRNAs differentially expressed between strongly haemolysed samples and non-haemolysed samples. Class comparison between the two

groups of plasma samples identified significant microRNAs up-regulated due to haemolysis, some of which are already known from the literature.

After haemolysis-related microRNAs exclusion, the class comparison analysis of circulating microRNAs performed on plasma samples of NeuT and BALB/c mice collected at early and late time points showed 28 up-regulated microRNAs at 12 weeks of age and 80 up-regulated microRNAs at 24 weeks of age in comparison to wt mice (Figure 33C). No down-regulated microRNAs emerged to be differentially expressed between NeuT and BALB/c plasma samples.

Importantly, among the predicted targets of up-regulated circulating microRNAs were found some the down-regulated genes in the BM of NeuT mice. In particular some of these genes, as reported above, are related to B cell signaling pathway and, in this line, some of circulating microRNAs have among their predicted targets B cell-related genes.

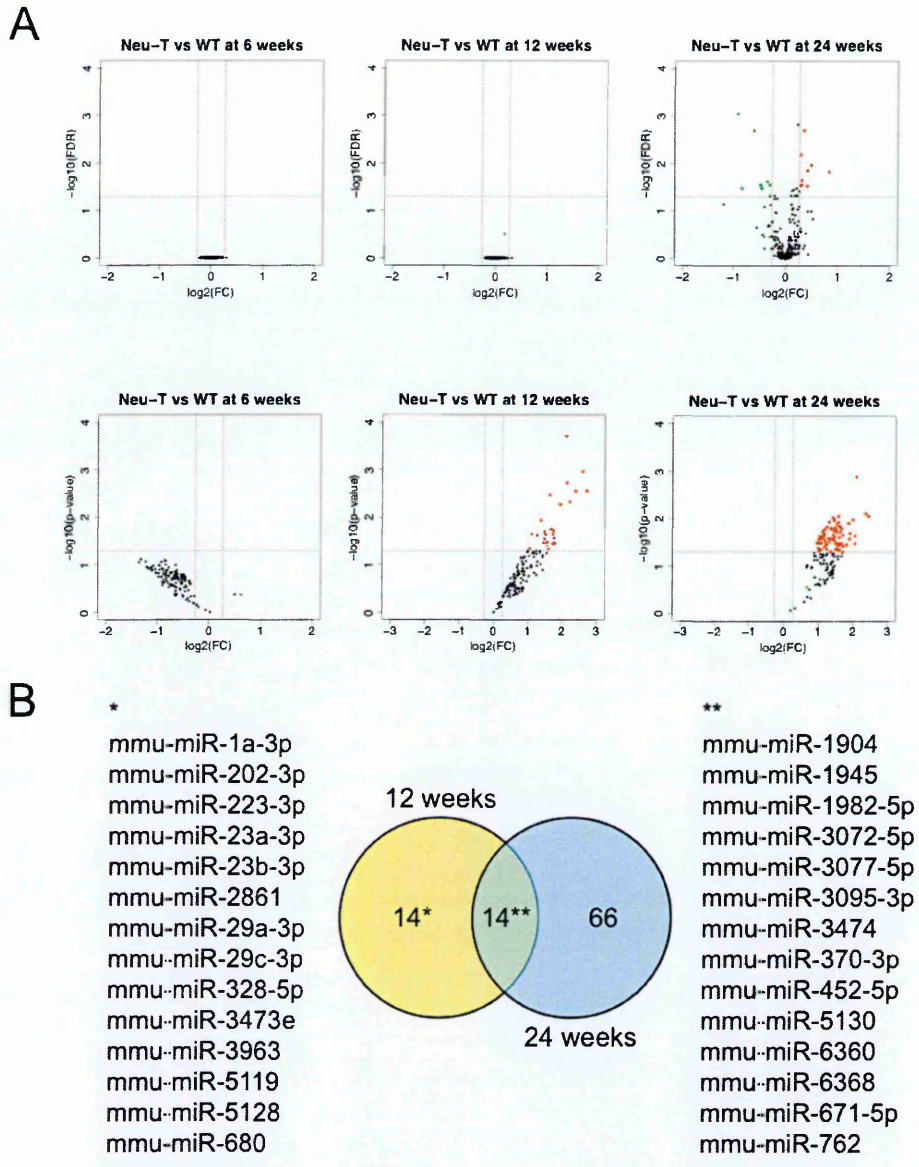


Figure 33 – BM and plasma differentially expressed microRNAs in NeuT and wt mice at different time points

A) Volcano plots of microRNAs profile in the BM of NeuT in comparison to BALB/c mice at 6, 12 and 24 weeks of age. B) Volcano plots of circulating microRNas profile in the comparison between NeuT and BALB/c mice at 6, 12 and 24 weeks of age and Venn diagram of differentially expressed microRNAs between 12 and 24 week samples. Differentially expressed microRNAs are represented in the Volcano plots as up-regulated (red) and down-regulated (green) dots.

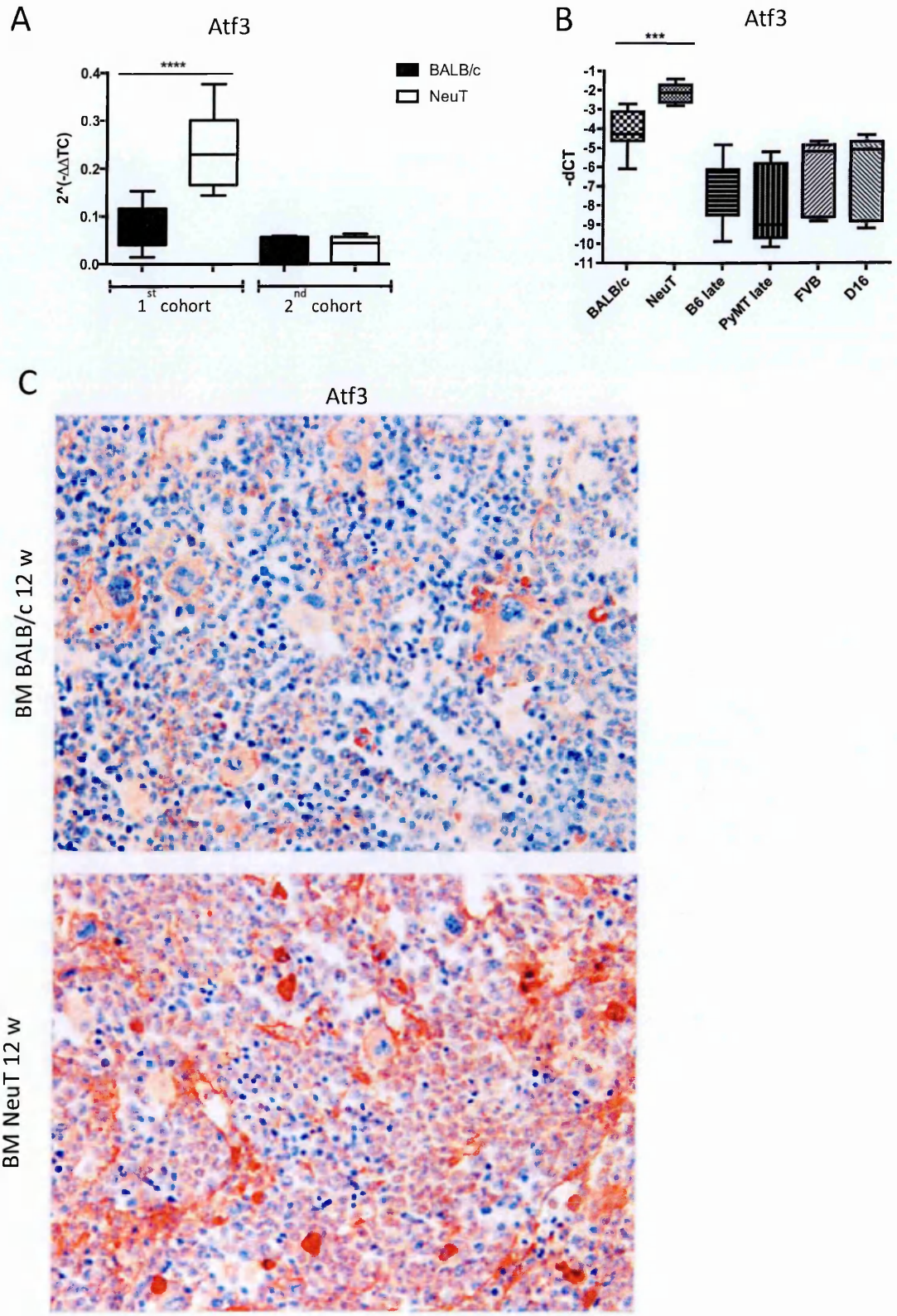
4.10 Atf3 is up-regulated in the BM myeloid/macrophages and in mammary glands of mice with early carcinomas

Among the genes more significantly up-regulated in the BM of NeuT mice with early cancer in comparison to wt mice and related with the inflammatory signature, the transcription factor Atf3 has been further validated by qRT-PCR on BM of NeuT mice at 12 weeks in comparison to BM samples of age-matched wt mice (Figure 34A). This modulation was confirmed in mice belonging to the first cohort, in which early cancers have been assessed, but not in the second cohort of mice in which lower disease severity have been observed in their mammary glands.

Being rapidly up-regulated in immune cells upon perturbation of homeostasis by various signals, Atf3 is an important regulator of immune response and cancer progression. The emerging link between CXCL12 and Atf3 in the ECM (Buganim, Madar et al. 2011) prompted us to further analyse this gene and the presence of the protein in the bone marrow tissue. Indeed, in situ immunohistochemical analysis confirmed Atf3 modulation in the granulocytic myeloid cells/macrophages of BM samples from 12 and 24 weeks transgenic mice in comparison to matched controls (Figure 34C). This result further confirmed our hypothesis of specific myeloid subsets undergoing expansion and re-localization within BM stromal niches upon sensing of "danger"-related signals from incipient malignancies.

The presence of Atf3 protein was also assessed in the mammary glands with early lesions in comparison to normal tissue. Interestingly, its expression was found to be more abundant in samples with early carcinoma and associated to myeloid/macrophage cells, in concordance with the expression in the BM parenchyma of mice with similar primary lesions (Figure 34D).

To assess whether Atf3 was also up-regulated in other breast cancer models, we evaluated the expression of this gene on BM from PyMT mice of 12 and 24 weeks of age and on BM from huHer2Δ16 mice of 9 and 24 weeks. Results obtained for both early and late time points, differently from the data obtained in the NeuT model, did not show any modulation in the expression between tg and wt mice and the overall level of expression in both models was much lower than in NeuT samples (Figure 34B).


A
Atf3
****
BALB/c
NeuT
2^(-ΔΔTC)
0.4
0.3
0.2
0.1
0.0
1st cohort
2nd cohort
B
Atf3
***
-dCT
-1
-2
-3
-4
-5
-6
-7
-8
-9
-10
-11
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BM BALB/c 12 w
BM NeuT 12 w

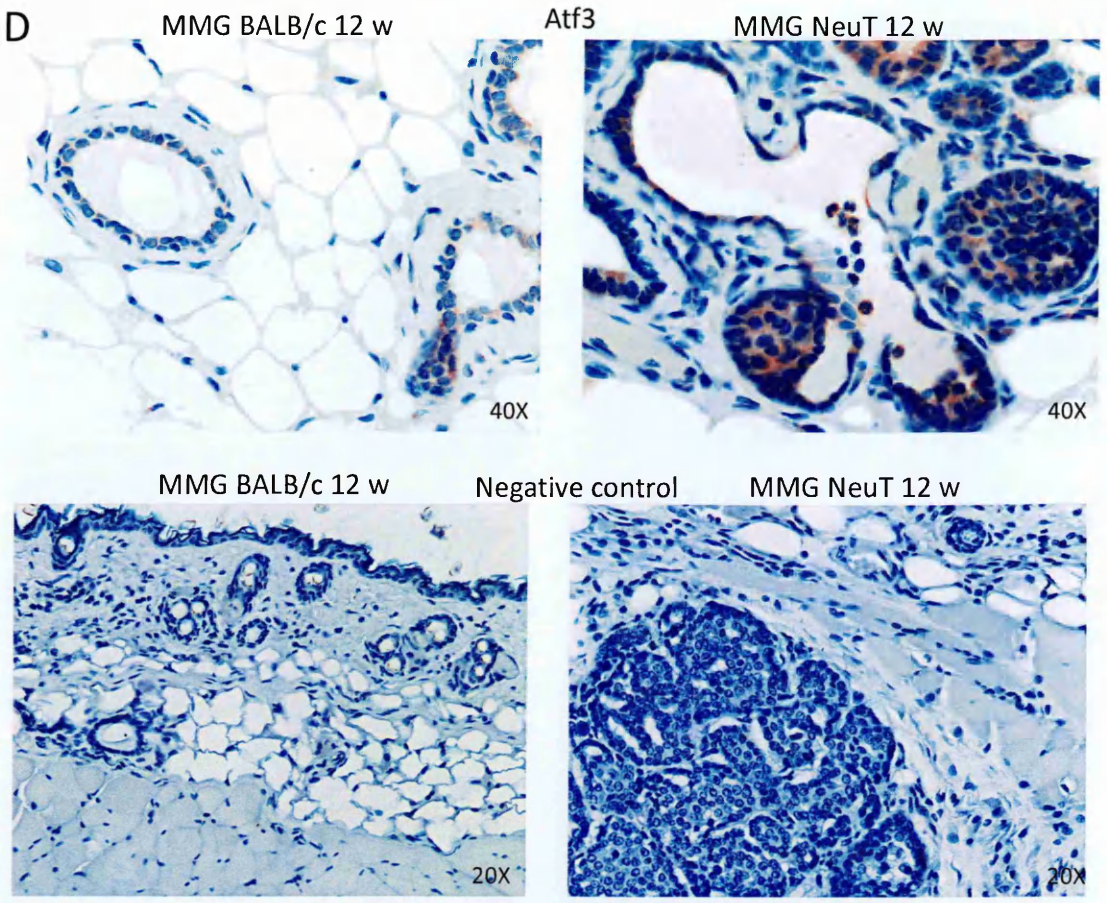

Figure 34 – Evaluation of Atf3 expression on BM and mammary glands (MMG) of NeuT mice and in BM of PyMT and huHer2Δ16 mice

A) Atf3 relative expression by qRT-PCR between validation (first cohort) set and discovery set (second cohort) in the BM of BALB/c and NeuT mice at 12 weeks of age. B) Atf3 relative expression in the BM of NeuT, PyMT, huHER2Δ16 (D16) and related controls (BALB/c, B6 and FVB) at 12 weeks of age. C) IHC for Atf3 in BM parenchyma of BALB/c and NeuT mice at 12 weeks of age. Images are representative of the discovery set. D) IHC for Atf3 on mammary glands tissue (MMG) collected form BALB/c and NeuT mice at 12 weeks of age in the discovery set.

4.11 Effects of Atf3 overexpression in engineered macrophage cell lines and in BM progenitor cells

The expression of Atf3 has been evaluated by WB and qRT-PCR upon infection of different macrophage cell lines (J774, RAW and MT2) with lentivector that carrying Atf3 under the control of the myeloid specific promoter CD68 (Figure 35A). All infected macrophage cell lines expressed a higher mRNA amount of Atf3 in comparison to wt counterparts although at different levels (Figure 35B). This result could be explained by the different expression of CD68 in the three cell lines, that was higher in J774 cells in comparison to RAW and MT2 cell lines (Figure 35C). The expression of some Atf3 target genes such as MMP-9 and IL-6 have been evaluated in this macrophage cell line. As expected infected macrophages, overexpressing Atf3 (Figure 35D), have shown to modulate also some of Atf3-related targets. Indeed, MMP-9 is found to be up-regulated in J774CD68Atf3 in comparison to wt J774 cell line; and Il-6 is found down-regulated in tg macrophages (Figure 35E).

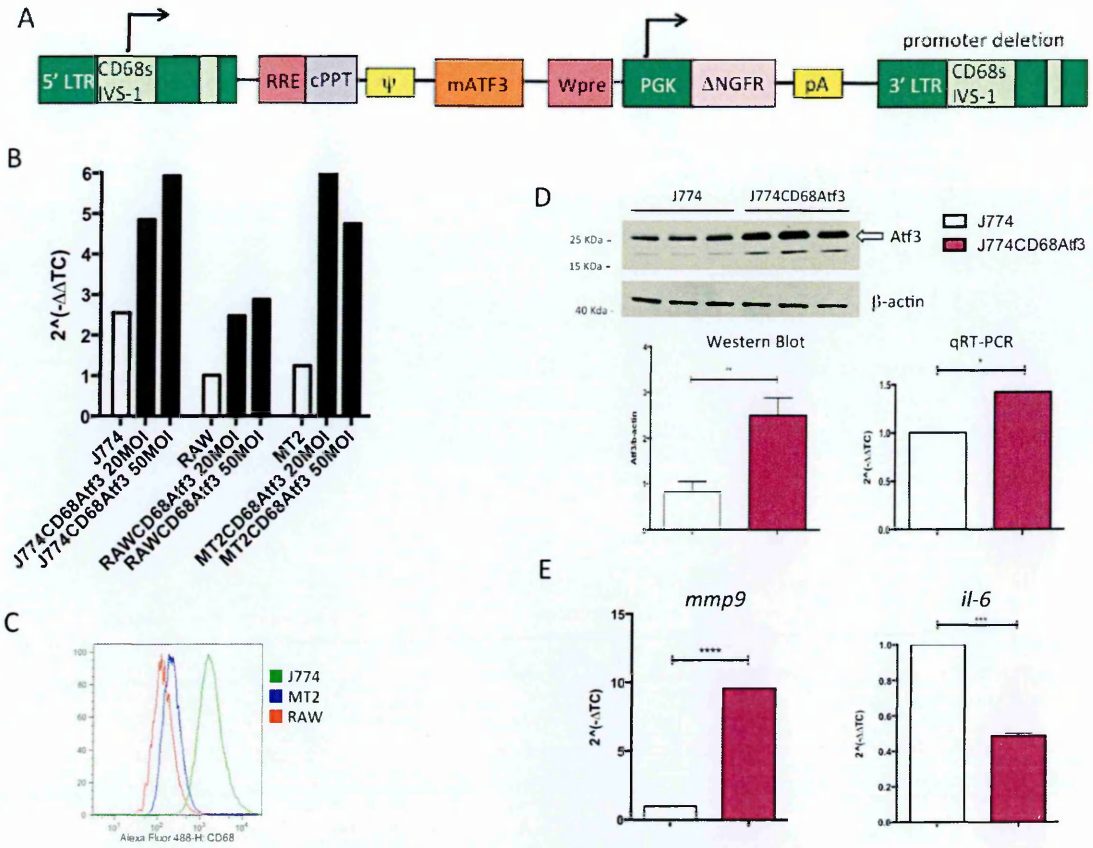

Figure 35 – Overexpression of Atf3 in macrophage cell lines by means of lentiviral infection

A) Schematic representation of pRRLsinCD68ATF3.WprePGKNGFR lentiviral vector. B) Atf3 relative expression of normal J774, RAW and MT2 macrophage cell lines or infected with lentivirus encoding for Atf3 at different MOI. C) FC analysis for CD68 in normal J774, RAW and MT2 macrophage cell lines. D) Atf3 protein quantification by Western blot analysis in normal J774 and infected J774CD68Atf3 cell line and relative quantification of Atf3 by Western blot and qRT-PCR. E) mmp9 and il-6 relative expression by qRT-PCR in J774 and J774CD68Atf3 cell line.

In order to investigate weather Atf3 could have a role in driving the expansion of BM hematopoietic progenitors, colony forming unit (CFU) assay has been performed in BM-derived lineage negative cells (lin^-) infected or not with Atf3 expressing vector, namely lin^-CD68Atf3 and lin^-, respectively. The number and types of colonies provide information about the frequency and types of progenitors present in the original cell population and their ability to proliferate and differentiate to specific subsets. After assessing by FC the expression of hNGFR in infected cells and selecting, by antibody-conjugated magnetic separation, the positive fraction, lin- and lin-CD68Atf3 cells were plated in suitable semi-solid matrix and CFUs count has been performed after 8, 11 and 13 days. Interestingly, after 8 days of culture an increase in CFU-M (colony-forming units-macrophage) was observed in CD68Atf3lin^- cells in comparison to wt counterparts. Such increase was maintained at 11 days and was paralleled by an expansion of CFU-G (colony-forming unit-granulocyte) in CD68Atf3lin^- cells. After 13 days these increments became more significant and the total CFU-GM (colony forming unit-granulocyte, macrophage) was also increased. This result suggests that the overexpression of Atf3 in early BM progenitors could favour the expansion towards granulocyte/macrophage progenitors in agreement with the high Atf3 expression found in these cell subsets in the BM parenchyma of NeuT mice (Figure 36).

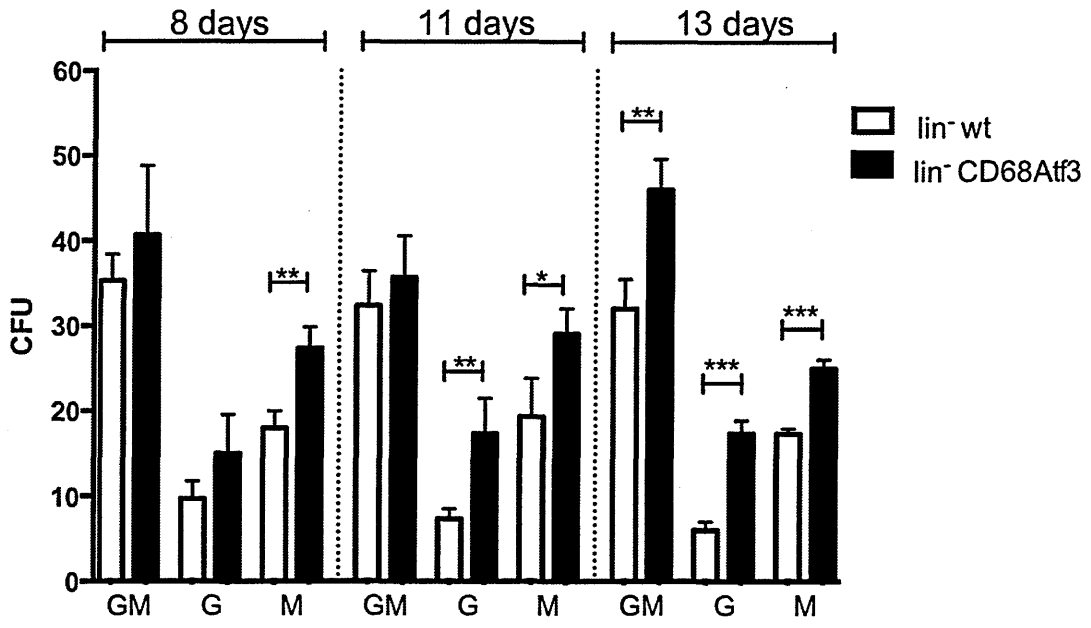

Figure 36 – Colony forming unit assay on derived BM lineage negative cells

Cell counts of colony forming unit (CFU) assay of BALB/c derived lin$^-$ cells infected or not with CD68Atf3 expressing vector. Cell counts were performed at 8, 11 and 13 days on CFU-GM, CFU-G and CFU-M by optical microscopy.

5 Discussion

The study of the tumour microenvironment and of its role in tumour development and progression has been mainly focused on local districts directly adjacent to primary or metastatic tumours. In this context a variety of cell types, such as stromal fibroblasts, endothelial cells and immune cells have been studied to understand how they foster tumour growth and dissemination. It is also now clear that tumour infiltrating immune cells are recruited from distant sites, primarily the BM, and actively contribute to the tumour promoting microenvironment of primary and metastatic tumours (Chantrain, Feron et al. 2008)

The main hypothesis of the project was that, during tumour development, starting from pre-malignant stages, cells undergoing neoplastic transformation and nearby stroma cells undertake a vicious cross-talk with the BM hematopoietic and stromal parenchyma. This cross-talk is oriented to the instruction of the BM towards tumour-promoting haematopoiesis.

As a tumour recruits from the BM immune cells that in turn contribute to the local inflammatory and angiogenic reaction associated to malignant transformation and progression (Coussens and Werb 2002) it is conceivable that the BM would undergo important alterations during an on going distant transformation, even at the very early phases.

Taking advantage of animal models of mammary carcinogenesis, within this study we indeed demonstrated that the BM actually represents an early sensor of incipient malignant transformation at distant sites.

To define and characterize in details the features of BM modification associated to distant transformation, different experimental methodologies were adopted, which included FC analysis on precursor and differentiated hematopoietic populations in the BM, spleen and PBL, BM histopathological and immunophenotypical analysis of hematopoietic and stromal mesenchymal components, gene profiling of the BM and microRNA profiling of BM and plasma samples and histopathology of mouse mammary glands. Of note, all of the analyses mentioned above were carried out on the same mice in order to maximize the comparability of the results obtained by the different methodologies. In addition, this comparative approach could allow correlating a precise modification in the transforming tissue to the response induced in the BM of the same animal, and potentially to circulating signals that can be ideally detected in the peripheral blood.

Firstly, the attention has been addressed to relatively advanced stages of tumour progression, namely 24 weeks of age in the spontaneous transgenic NeuT breast cancer model that also served as positive reference for the subsequent analyses on earlier stages.

In 24 weeks old NeuT tumour-bearing mice a profound modification in the composition and spatial arrangement of the hematopoietic populations have been identified in comparison to healthy mice, confirming that the BM clearly responds to a distant transformation tuning its haematopoiesis.

The accumulation of $CD11b^+$ cells in the tumour microenvironment at overt tumour stage and in the periphery (BM, spleen and PBL) of NeuT mice has been previously demonstrated by our group (Melani, Chiodoni et al. 2003). We confirmed here the FC results that were corroborated by, histological analysis on

the BM parenchyma where myeloid cells with an immature morphology have been detected.

Interestingly, we observed a concomitant and significant reduction of B cells, as well as a displacement of lymphoid cells and a reduction in the erythroid pools. The contraction of the B cell compartment was not confined to NeuT mice, but was also detected in the MMTV-PyMT model.

Accordingly, in the latter model, a complete block in B cell lymphopoiesis, dependent on tumour burden, has been recently reported by another group (Moreau, Mielnik et al. 2016). In the same model, Casbon et al. showed a skewing toward myeloid cell production in BM of mice with progressive disease that was paralleled by a decrease in erythropoiesis (Casbon, Reynaud et al. 2015).

At late time point, the integration of the precise quantification of different hematopoietic subpopulations achieved by FC analysis with the *in situ* data of their numbers and topographical distribution confirmed the robustness of such combined approach.

The modulations observed in differentiated cells reflected the expansion of their progenitors; indeed the BM of tumour-bearing mice displayed an increase in GMP and a reduction of MEP, phenomena that are consistent with the expansion of myeloid fraction and the reduction of erythroid cells, respectively.

The BM modifications occurring in tumour-bearing mice also were confirmed using genomic approaches, through GEP and relative analyses on BM cells, which allowed an in depth view of the molecular processes involved. Indeed GEP of BM from mice with advanced cancers displayed hundreds of genes differentially expressed in comparison to wt mice. The top biological processes characterizing

up-regulated genes in NeuT mice are consistent with innate immune and inflammatory response-related programs. Down-regulated genes in these mice comprise processes belonging to adaptive immune response, lymphocyte differentiation and activation programs and B cell signalling.

Accordingly, the presence of an active inflammatory process is well known to be linked to tumour development and it is confirmed by cases in which cancer arose from chronic inflammation status (Mantovani, Allavena et al. 2008). In addition, a dampening in the immune system response is also reported to be adopted by the tumour to grow, evading immunosurveillance (Dunn, Old et al. 2004).

In addition, it has been applied for the first time in a mouse setting the human-based analytic tool Cibersort. (Newman, Liu et al. 2015), which confirmed a contraction of B lymphocytes and an accumulation of neutrophils in NeuT mice in comparison to wt mice, making this tool, at least in part, reliable in mouse settings.

The lists of up- and down-regulated genes in the BM of NeuT mice were used to build a "late BM gene signature", which was further validated not only in an independent cohort of NeuT tumour-bearing mice, but also in two different murine breast cancer models, namely the PyMT and the huHER2Δ16, indicating that BM transcriptional changes associated to overt carcinogenesis are shared by different mammary tumour models despite distinct driving oncogenes and genetic backgrounds.

Starting from these evidences in tumour-bearing mice, the analyses have been performed at earlier time points of tumour progression, namely 6 and 12 weeks of age. At these stages, the modulation of immune cells analysed by FC followed a similar trend although not as significant as the one occurring at later stages. The

modifications in BM parenchyma through histopathology examinations were more evident and consistent with the initial hypothesis. Indeed, an increase in neutrophils and myeloid immature cells at the expense of erythroid pools characterized the BM of these mice. In this scenario, mesenchymal cells also expanded in both osteoblastic and vascular niches and displayed an upregulation of CXCL12 expression. Consistent with this change in BM mesenchymal cell phenotype was the enrichment in CXCR4-expressing myeloid cells in the same BM areas. Overall, these results suggest that the early modifications occurring in the BM parenchyma at premalignant phases are mainly related to an innate immune cell subset, specifically myeloid cells that are enriched at the expense of other resident populations, being favoured in their relocation within BM erythroid and lymphoid niches by the CXCL-12/CXCR-4 axis. Accordingly, this axis was reported to be crucial in the mobilization of BM-derived cells to the sites of transformation (Levesque, Hendy et al. 2003).

GEP analyses at early time points displayed different gene modulation between discovery and validation set. Such discrepancy could stem from mouse individual variability, in the onset and extension of nascent lesions of NeuT mice. Indeed, such variability has been confirmed by in depth histopathological analysis of the mammary glands of each mouse of the two cohorts and allowed us to build an *ad hoc* adjusted score based on histopathological parameters to reclassify individuals independently from their age. Since the progression to *in situ* carcinoma was identified as a crucial event to be correlated with BM phenotypic and molecular changes, the "adjusted score" was built mainly considering the contribution of the grade/extension of dysplasia and of grade/extension of early carcinoma.

Re-classifying the early lesions on 4 histopathological categories (normal, mild/moderate dysplasia, severe dysplasia/early carcinoma) allowed analysis of BM samples, with a higher resolving power, in order to identify which tissue transition has the major role in terms of differentially expressed transcripts in the BM. Eighteen genes were found differentially expressed between BM of mice with early carcinoma and BM of control mice.

The commonalities in the processes characterizing the BM during early and late phases of breast tumour development provides the link between the early modification in the transforming tissue and a precise BM transcriptional pattern. In fact, the late BM signature very efficiently separates early BM samples of mice that have normal/mild dysplasia in their mammary glands from those with severe dysplasia/early carcinomas. Notably, even if no differences in transcripts have been observed with class comparison analysis between NeuT and BALB/c mice at 6 weeks, the late BM gene signature is able to clearly distinguish NeuT from BALB/c samples at this very early time point.

Even if GEP at 6 weeks does not reveal any statistically significant gene differentially expressed, GSEA further supports the hypothesis that BM responds very early to a peripheral perturbation undergoing stromal changes. In fact ECM- and stroma remodelling-related genes were found enriched in BM from 6-week NeuT mice. Recent pieces of evidence suggest an important role of collagens and other matricellular proteins in tuning both BM and TME. Indeed, some of these genes are described to regulate, in the stroma of breast cancer patients, the remodelling of tumour microenvironment by structural organizing the ECM components (Triulzi, Casalini et al. 2013). This may suggest that modification in

ECM proteins and stromal components occurring in the primary tumour could be similar to those observed distant organs sensing the tumour development such as BM.

In order to identify the signals potentially responsible for the induction of the early BM changes associated to incipient neoplasia, analysis of both BM and circulating microRNAs was performed and identified, in the BM, at late time point, among the mostly up-regulated microRNAs mir-21a-5p and miR-146b-5p, both already implicated in breast cancer. Indeed, miR-21 has been shown, in human settings, to function as an oncogene and to be overexpressed in breast carcinoma tissues mediating cell survival, proliferation, extravasation and metastasis (Iorio, Ferracin et al. 2005). MiR-146, beside being described to be relevant in innate immunity and inflammation, (Taganov, Boldin et al. 2006), was also demonstrated to be involved in breast carcinogenesis where it correlates with poor prognosis.

Among microRNAs down modulated in the BM, miR-92 is also involved in breast cancer, where its down-regulation is associated with aggressive BC features (Smith, Baxter et al. 2015).

The analysis of circulating microRNAs significantly modulated at both late and early time points showed several microRNAs as differentially expressed between NeuT and wt mice, specifically 28 microRNAs up-regulated at 12 weeks and 80 microRNAs up-regulated at 24 weeks. Among these microRNAs 14 were in common between the two time points. Some of these microRNAs were already described in the literature to modulate stroma related proteins (mir-29s, mir-328, mir-671) or regulating cell cycle, adipogenesis and proliferation and/or already associated with breast cancer features in human settings (Tan, Fu et al. 2016).

Importantly, among the predicted targets of up-regulated circulating microRNAs were found some the down-regulated genes in the BM of NeuT mice. In particular the majority of these genes are related to B cell signalling pathway and, in this line, some of circulating microRNAs have among their predicted targets B cell-related genes.

Among the most up-regulated genes in the BM of NeuT mice with early carcinomas, we focused our attention on Atf3, which was already identified as differentially expressed in NeuT and BALB/c mice in the discovery set at 12 weeks of age. Its role in cancer has been already described, as it was found highly expressed in the stroma of human breast cancers and correlating with poor prognosis (Wolford, McConoughey et al. 2013) and associated to CXCL12 enriched areas in the ECM of human tumours (Buganim, Madar et al. 2011).

In both BM and mammary glands of NeuT mice with early carcinomas a high expression of Atf3 was found in myeloid/macrophage cells. Hypothesizing a putative role of Atf3 in participating to the process regulating myeloid/macrophage mobilization from the BM, we decided to address its role in NeuT tumour development and progression by over-expressing it in cells of the myeloid lineage. Preliminary data on Atf3-overexpressing macrophages indicate a negative control on the production of the pro-inflammatory cytokine IL-6, in line with the situation likely occurring in the BM in presence of early carcinomas when inflammatory processes and danger-related signals are activated. Furthermore upon over-expression of Atf3 we observed an induction of MMP9 production, which is known to have a crucial role in the expansion and function of MDSCs (Melani, Sangaletti et al. 2007). Preliminary data of overexpression of Atf3 in BM progenitor cells

showed an increased potential in colony formation and in particular in the induction of macrophage/myeloid cell subset.

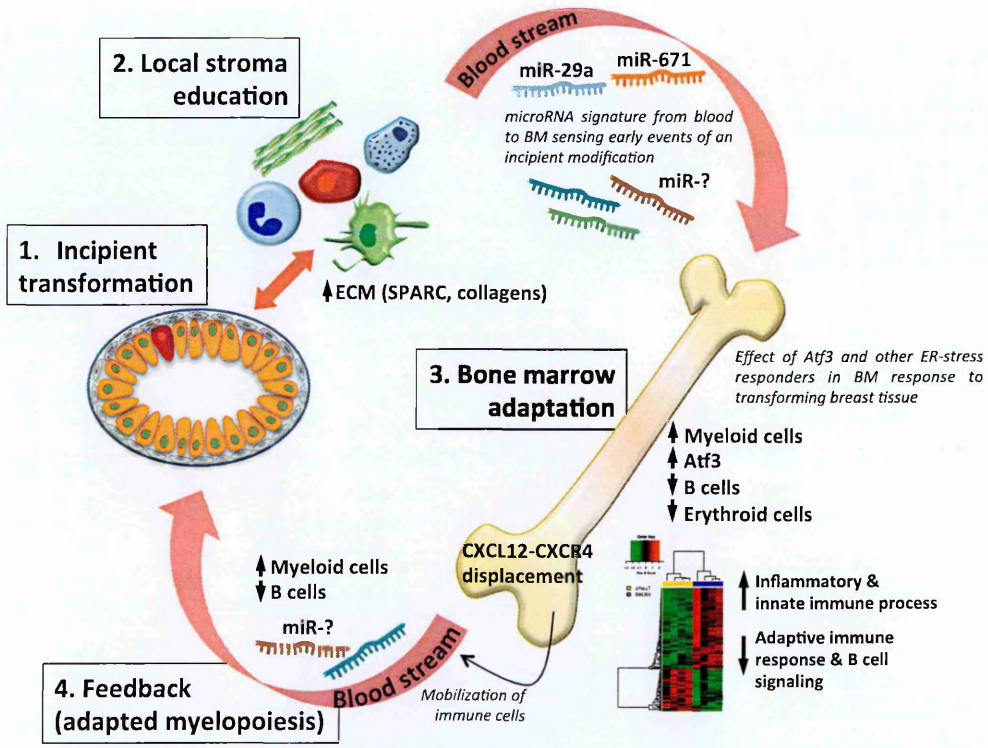

Figure 37 – Schematic overview of the cross-communication between an incipient mammary tumour and the BM

The presence of an incipient tumour induces a series of modification in the stroma, such as modifications in the ECM composition, that are reflected in the variations of circulating microRNAs. The BM reacts to these variations with an increase in stress response signals (Atf3 and inflammatory processes), a decrease of B cells and an increase in myeloid cell number. This adaptation of the BM to the presence of a nascent tumour activates a feedback response consisting in the mobilization of myeloid cells in the blood stream and microRNAs that can tune tumour growth.

6 On going studies and future prospective

In light of the role of Atf3 in inflammation and in TME we have also planned to assess the biological activity of this protein in NeuT model either by silencing or forcing its expression in cells of BM origin. In particular, in order to evaluate the effect of such modifications in terms of tumour development and of alteration of the BM environment we will transplant Atf3 overexpressing BM progenitor cells into irradiated BALB/c and NeuT mice. The transplantation of transduced BM cells into lethally irradiated BALB/c recipients will allow assessing whether the hematopoietic cell expression of Atf3 is sufficient to establish the BM variations we have detected in NeuT-bearing mice in absence of an on going neoplastic transformation. On the other hand BM transplantation with engineered BM cells of young NeuT recipient mice will define whether the forced expression of Atf3 specifically in the myeloid compartment is sufficient to modify tumour outcome.

Moreover, in light of the results obtained by microRNA profile that lead to the identification of circulating microRNAs differentially expressed between tumour-bearers and wt mice, we will design specific experiments to define the biological and functional relevance of some specific microRNAs we have found to be particularly relevant in our setting.

In addition, in order to obtain more precise and significant data all the analysis performed for microRNA profiles will be re-evaluated on the samples after the re-classification for the severity of the disease, as performed for GEP data.

Moreover, the differential expression of some of the microRNAs modulated in NeuT mice will be checked also in plasma sample of breast cancer patients

pointing out the possibility that the definition of a mouse-derived microRNA signature could be transferred into human breast cancer series.

7 Publications

Sangaletti S, Tripodo C, Sandri S, Torselli I, Vitali C, Ratti C, Botti L, Burocchi A, Porcasi R, Tomirotti A, Colombo MP & Chiodoni C (2014) "Osteopontin shapes immunosuppression in the metastatic niche." Cancer Res. **74**, 4706–19

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